AWARD NUMBER: W81XWH-14-1-0197

TITLE: FLT-PET/CT as a Biomarker of Therapeutic Response in Pemetrexed Therapy for Non-Small Cell Lung Cancer

PRINCIPAL INVESTIGATOR: Sharyn I Katz, MD, MTR

CONTRACTING ORGANIZATION: University of Pennsylvania Perelman School of Medicine Philadelphia, PA 19104

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14. ABSTRACT: Pemetrexed(PEM), a standard therapy for non-squamous non-small cell lung cancer (NSCLC), inhibits the de novo thymidine pathway, resulting in a transient burst of metabolism through the salvage pathway, an effect detected as a "flare" of activity by 18F-thymidine (FLT)-PET. FLT is a reliable biomarker of proliferation, and post-therapeutic changes in tumor FLT avidity predicts therapeutic response in a range of malignancies. However, FLT as a measure of therapeutic response in NSCLC is not well studied. Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC with the following specific aims: Specific Aim 1: To determine the optimal timing to measure FLT "flare", an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model. Specific Aim 2: To conduct a proof-of-concept clinical study of FLT flare and FLT-measured changes in tumor proliferation at 2 weeks of therapy as predictors of NSCLC response to PEM. During the research period, we have determined that the optimal time to observe FLT flare in vivo is at 2 hrs following therapy start. This data has been translated to an open clinical trial of FLT-PET as a biomarker of therapy response in NSCLC proposed in Specific Aim #2.

15. SUBJECT TERMS
FLT-PET, biomarker, therapy response, Non-small cell lung cancer,

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Perelman
School of Medicine
UNIVERSITY *of* PENNSYLVANIA

Sharyn I Katz, MD, MTR
Assistant Professor,
Division of Thoracic Radiology

8/9/17

Elizabeth Yu, PhD
Science Officer, LCRP and TSCRP
Dear Ms. Cardoza:

This letter is in response to your request for revisions of the final technical report regarding Proposal Log Number LC130313, Award Number W81XWH-14-1-0197, HRPO Log Number A-18540. Below are answers to the stated questions with the required supporting document attached. Please to feel free to contact me with any further questions.

1. ***Firstly, MRMC reporting requirements (http://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting) mandate that final reports be a comprehensive reporting of all work conducted during the award period of performance. This means the summary included under the accomplishments should include a description of work completed under all tasks, not just those focused on in year 2.***

Response: This information has been added to the summary of accomplishments as directed.

2. Secondly, you must include representative data supporting all claims
in your summary. This will include data from the mouse work in Aim 1, and any clinical data from Aim 2 you are able to provide.

Response: This information has been added to the summary of accomplishments as directed. Of note, the pre-clinical work has resulted in 2 papers, both published in Oncotarget and in press.

3. ***Additionally, deviations from the approved SOW, including your decision to eliminate histologic analyses in Aim 1 and major delays in clinical trial enrollment should be fully justified. As written, it is not clear what efforts were made to improve enrollment when difficulties arose, and why only 1 patient was enrolled between March 2016 and the close of the award.***

Response: The explanation on the SOW have been edited to further explain why the histological analysis was not performed on the explants from the *in vivo* studies and why clinical trial accrual was slower than anticipated.

Sincerely,

Sharyn I. Katz, M.D., M.T.R.

TABLE OF CONTENTS

10. INTRODUCTION:

Pemetrexed(PEM), a standard therapy for non-squamous non-small cell lung cancer (NSCLC), inhibits the de novo thymidine pathway, resulting in a transient burst of metabolism through the salvage pathway, an effect detected as a "flare" of activity by 18F-thymidine (FLT)-PET. FLT is a reliable biomarker of proliferation, and post-therapeutic changes in tumor FLT avidity predicts therapeutic response in a range of malignancies. However, FLT as a measure of therapeutic response in NSCLC is not well studied. Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC with the following specific aims: Specific Aim 1: To determine the optimal timing to measure FLT "flare", an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model. Specific Aim 2: To conduct a proof-of-concept clinical study of FLT flare and FLT-measured changes in tumor proliferation at 2 weeks of therapy as predictors of NSCLC response to PEM. During the research period, we have determined that the optimal time to observe FLT flare in vivo is at 2 hrs following therapy start. This data has been translated to an open clinical trial of FLT-PET as a biomarker of therapy response in NSCLC proposed in Specific Aim #2.

11. KEYWORDS:

FLT-PET, biomarker, therapy response, Non-small cell lung cancer,

12. ACCOMPLISHMENTS:
What were the major goals of the project?

Major Task 1: Training and educational development in lung cancer research	Projected Months	Percentage of Task Currently Completed (%)	Anticipated Time to Completion (months)	Comments
Subtask 1: Meet biweekly with Drs. Schnall (primary mentor) and Albelda (co-mentor) and monthly with co-mentors Drs. Mankoff and Langer	1-24	100%		I am continuing to meet with my mentoring team as scheduled.
Subtask 2: Attend weekly meetings and present research at the Penn Molecular Imaging Research Group	1-24	100%		I am continuing to attend these meetings and to present research.
Subtask 3: Attend monthly meetings and present research at the Tobacco and Environmental Carcinogenesis Clinical Trials Working Group	1-24	0%	n/a	This working group no longer has regular meetings

Subtask 4: Attend relevant seminars and meetings of the Abramson Cancer Center Lung Center of Excellence	1-24	100%		These seminars and meetings are ongoing, and I am continuing to attend.
Subtask 5: Attend annual ACRIN-ECOG and meetings for the Young Investigator Initiative for Conduct of ACRIN Ancillary Research	1-24	100%		I am continuing to attend these meetings.
Subtask 6: Attend annual World Congress of Molecular Imaging and one Keystone Symposium on cancer biology	1-24	0%	1-12	I did not attend a WMIC or Keystone meeting. Instead, I did attend and present this study data at the AACR 2016 meeting.
Subtask 7: Complete formal coursework at UPENN	1-24	0%	1-12	I still need to do the proposed Penn coursework
Milestone(s) currently Achieved: • *Integration into Thoracic Core Committee of the ACRIN-ECOG organization* • *Mature clinical research skills through formal meetings, data presentation and networking.*				*In the past two years, I have attended the fall and spring working group meetings for ECOG-ACRIN and have given research talks to the ECOG-ACRIN Thoracic Core committee. As a result, I have developed new collaborations and new research directions*

Research-Specific Tasks:

Specific Aim 1: To determine the optimal timing to measure FLT "flare" as an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model.		100%		
Major Task 1: To validate xenograft chemotherapeutic regimen of PEM-induced FLT flare in a NSCLC xenograft mouse model.	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Obtain USAMRMC Office of Research Protections (ORP) approval for IACUC protocol(institutional approval anticipated in advance of the start of the grant award period).	1-3	100%		
Subtask 2: NSCLC xenograft trial of PEM + cisplatin to confirm efficacy of therapeutic regimen. Cell line used: H460 [Gift of Dr. Albelda] Mice: 6 wk old mice *nu/nu* (Strain#088, Charles River Labs); (Total mice: 8 mice/group x 2 groups = 16 mice)	4-5	100%		
Subtask 3: Histologic analysis of excised tumors (CHOP Pathology Core)	5	0%	1-3	We determined that histologic analysis was not necessary for publication. The correlation between FLT avidity and proliferation is well established in the literature therefore we did not feel that it was necessary

				to demonstrate this correlation. In addition, we felt that the robust tumor growth inhibition was adequate to demonstrate therapy response and histological analysis to demonstrate apoptosis within the tumor was unnecessary to justify our conclusions
Subtask 4: Statistical Analysis of data	5	100%	1-3	
Major Task 2: Characterization of kinetics of FLT "flare" in xenograft model	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 2: Examine FLT "flare" kinetics in PEM-sensitive NSCLC xenografts. This will be done for 3 PEM-sensitive cell lines. Cell lines used: H1299, H460, H1264 [Gift of Dr. Albelda] Mice: 6 wk old mice *nu/nu* (Strain#088, Charles River Labs); (Total mice: 3 cell lines x 8 mice/group x 2 groups x 5 time points = 240 mice) FLT/PET: FLT (250 µCi/FLT-PET scan). Resources available through the UPENN Dept. of Radiology Cyclotron and Small Animal	6-9	100%	1-3	

Imaging Facility (SAIF).				
Subtask 2: Histologic analysis of excised tumors (CHOP Pathology Core)	9	0%	1-3	We determined that histologic analysis was not necessary for publication. The correlation between FLT avidity and proliferation is well established in the literature therefore we did not feel that it was necessary to demonstrate this correlation. In addition, we felt that the robust tumor growth inhibition was adequate to demonstrate therapy response and histological analysis to demonstrate apoptosis within the tumor was unnecessary to justify our conclusions
Subtask 2: Statistical Analysis of data	9	100%	1-3	
Subtask 3: Submission of manuscript for publication	10-13	100%	3-6	Data published in Oncotarget in 2016
Subtask 4: Present data at a national meeting	13-24	100%	6-12	Data presented as a poster at the 2016 annual

				meeting of the AACR
Milestone(s) Achieved: • *Through in vitro and completed in vivo data, we have determined that the optimal time to observe FLT flare in vivo is at 2hrs following therapy start.*				The pre-clinical aim is complete and we have successfully determined the optimal timing to observe the FLT flare effect in a mouse model of NSCLC. In addition, this data has been successful published and presented at a national meeting.
Specific Aim 2: Proof-of-concept clinical study of FLT therapeutic response prediction of NSCLC response to PEM-based therapy.		10%		
Major Task 2: Prepare for opening of clinical study	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Obtain USAMRMC ORP HRPO approval of IRB protocol (IRB approval anticipated in advance of award start)	1-3	100%		
Subtask 2: Finalize choice of clinical research assistant (CRA) from available pool of Dept. CRAs.	1-3	100%		
Subtask 3: Prepare for study opening (organize study binders, define clear study algorithm and review with study personnel)	3-12	100%		

	Months	Percentage of Task Currently Completed (%)	Anticipated Time to Completion (months)	Comments
Subtask 4: Determine the optimal timing to observe the FLT "flare" from preclinical obtained through Spec Aim1. If pre-clinical data is indeterminate, FLT flare will be measured at 1hr following infusion of PEM, similar to what has been suggested for 5-FU[10] and capecitabine[12].	9	100%		
Milestone(s) Achieved: • *Select and organize study personnel* • *Finalize regulatory documents and clinical trial design*		100%		
Major Task 3: Clinical study accrual	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Begin patient recruitment. CRA to consent patients in offices of the Division of Thoracic Oncology in collaboration with the patient's oncologist. Anticipated quarterly accrual: 20	10-14	100%		
Subtask 2: Complete study accrual and close study enrollment.	14	10%		
Subtask 3: Complete progression-free survival data	11-20	5%		
Milestone(s) Achieved: • *Begun patient accrual for clinical trial* • *Begun of follow-up data collection on progression-free survival*				To date, 3 of the total 20 anticipated patients have been enrolled into the clinical trial. The reason for the poor accrual is in part due to the shift of the field to

				immunotherapy. Additional factors include difficulty in engaging this population of terminal cancer patients at the diagnosis of their cancer as well as other clinical trial competing for the same patient population. In order to attempt to improve enrollment, a clinical research assistant was engaged to assist in identifying and approaching all new patients being seen at UPENN with a diagnosis with NSCLC and regular meetings were held with the oncologists in order to find improved means recruitment. In addition, the structure of the protocol was modified to image during the 2nd cycle of therapy in order to allow more time for patients

				to adjust to their diagnosis prior to approaching them for the imaging studies. In addition the reimbursement for patient participation in the study was increased Any patients that were found to meet the criteria for the study were approached and followed up. However despite all of these efforts, patients remained uninterested in a stand alone imaging trial that offered no therapeutic benefit and entailed imaging with an experimental agent.
Major Task 4: Clinical study data analysis	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Calculate CT RECIST data for 1st restaging	14-17	100%	9-11	Pt. 1 could not have RECIST measurements because she died prior to restaging.

				Pt.2 exhibited a partial response(-44%) and, interesting exhibited an FLT "flare" in response to pemetrexed therapy. Pt. 3 exhibited progressive disease (+48%) at 1st restaging and did not exhibit a FLT "flare"
Subtask 2: Calculate tumor FLT avidity from FLT-PET/CT scans. Performed on available UPENN clinical software.	11-16	100%	9-11	
Subtask 3: Statistical analysis of clinical study data	20-21	0%	9-11	Not felt to be useful with a sample size of 3 patients
Subtask 4: Submission of manuscript of proof-of-concept study data for publication	22-24	100%	11-12	Images from a patient scan from this clinical trial was included in the published manuscript in Oncotarget and served as a proof of principle example of the "flare" technique translated to the clinic.
Subtask 5: Presentation of data at a national meeting	21-24	100%	11-12	The accrual was too low to present the data

				from this clinical trial on its own although examples from the patients that were imaged were included in the poster presented at the AACR.
Milestone(s) Achieved: • *These tasks are anticipated to be completed over the course of the next year*				Since accrual to the study was very low, analysis of the data was not performed since it will not reach statistical significance with 3 patients.

What was accomplished under these goals?

1) <u>Major activities</u>: During the period of the 1st year of this grant, the majority of the preclinical aim, Specific Aim #1 was completed and the clinical trial proposed in Specific Aim #2 was opened in a time period similar as projected by the initial Statement of Work. During the 2nd year of this grant, the preclinical aims were completed, published and present at an international scientific meeting and accrual to the clinical trial continued although was much slower than anticipated primarily due to challenges in recruiting patients with a new diagnosis of terminal NSCLC to a stand alone imaging clinical trial with no clinical benefit.

2) <u>Specific objectives</u>: Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC. Our primary objective is to assess whether "FLTflare", a transient (<24hrs) burst of DNA salvage pathway activity induced by TS inhibition, is predictive of PEM therapeutic success. Our secondary objective is to determine whether post-therapeutic changes in tumor

proliferation, assessed by FLT-PET at baseline and at 2 weeks of PEM therapy, correlate with tumor response.

3) <u>Significant results or key outcomes:</u> During the 1st year of the grant, a preclinical model of human non-small cell lung cancer (NSCLC) was employed to study the kinetics of the pemetrexed induced FLT "flare". *In vitro* studies of 2 human NSCLC cell lines, H460 and H1299, revealed that the activity of the DNA salvage pathway, as measured by ^{3}H-thymidine labeling, peaks 2 hours **(Appendix 1, Fig. 1)** following the exposure of cells to pemetrexed. Furthermore this increased activity of the DNA salvage pathway appears to be the result of shift in state of TK1, the rate limiting step of the DNA salvage pathway, to a more activated tetramer state **(Appendix 1, Fig. 2)** and the translocation of ENT1 **(Appendix 1, Fig. 1 and 3)**, the cell surface nucleoside transporter responsible for thymidine entry into the cell, from the nucleus to the cell surface. We then examined the timing of the FLT flare *in vivo* in a xenograft mouse model of NSCLC confirmed that the FLT flare peaks at 2 hrs of exposure to pemetrexed **(Appendix 1, Fig. 4)**. These data have resulted in a manuscript that is currently published in Oncotarget (paper attached in the **Appendix 1**; PMID: 27655645) and was presented at the ACCR at the annual meeting in New Orleans, LA, in April 2016 (Abstract 4233, see **Appendix 3**). In year 2 of the grant, we further extended the preclinical study of FLT flare to assess whether the FLT flare is predictive of pemetrexed resistance. Resistance to TS inhibition by pemetrexed was induced in NSCLC cell lines H460 and H1299 through TS overexpression. TS overexpression was confirmed with RT-PCR and Western blotting and pemetrexed resistance confirmed with IC50 assays. The presence of a pemetrexed-induced thymidine salvage pathway "flare" was then measured using 3H-thymidine in both pemetrexed sensitive (H460 and H1299) and resistant (H460R, H1299R, CALU-6, H522, H650, H661, H820, H1838) lines *in vitro*, and validated with FLT-PET *in vivo* using H460 and H460R xenografts. Using this approach, we demonstrated that overexpression of TS induced pemetrexed resistance with IC50 for H460, H1299, H460R and H1299R measured as 0.141 μM, 0.656 μM, 22.842 μM, 213.120 μM, respectively. Thymidine salvage pathway 3H-thymidine "flare" was observed following pemetrexed in H460 and H1299 but not H460R, H1299R **(Appendix 2, Fig. 2)**, CALU-6, H522, H650, H661, H820 or H1838 *in vitro* (**Appendix 2, Fig. 3**). Similarly, a FLT "flare" was observed *in vivo* following pemetrexed therapy in H460 but not H460R tumor-bearing xenografts **(Appendix 2, Fig. 4)**. These data have resulted in a manuscript that is currently published in Oncotarget (paper attached in the **Appendix 2**; currently in press) and was presented at the ACCR at the annual meeting in Washington, DC, in April 2017 (Abstract 1783, see **Appendix 3**).

The clinical trial proposed in Aim 2 was opened in year 1 however was very slow to accrue. This was due primarily to the fact that patients with newly diagnosed terminal NSCLC were reluctant to participate in an experimental imaging trial offering no therapeutic benefit. During the period of the grant we accrued a total of 3 patients, with the following characteristics (1 female, 2 male, ages

63-67). All three patients had a histology of stage IV primary adenocarcinoma of the lung. After starting pemetrexed based therapy, the mean overall survival was 124 days (4.1 months). RECIST measurements were not possible for Patient 1 since she died 24 hrs following the start of therapy due to complications of pneumonia. This patient was not able to undergo FLT scanning on the day of pemetrexed therapy start due to availability of the PET scanner. Patient 2 exhibited a partial response to therapy (-44%) at 1st restaging following the start of pemetrexed-based therapy. Interestingly, this patient also exhibited an FLT "flare" of 45% increase in FLT avidity (SUVmax 5.41 to 7.91) over baseline. The images from this patient's FLT scan were published in Oncotarget (see Appendix 1). Patient 3 exhibited disease progression at re-staging (48% increase) and did not exhibit a FLT "flare" on the day of therapy start. All 3 patients had FLT scans 1-6 weeks following the start of therapy. No significant change was identified from baseline on those scans. Since the sample size is extremely small, no conclusions can be drawn at this point as to whether the FLT flare is predictive of therapy success in clinical therapy. However, through this pilot trial we have thus far shown feasibility of this strategy in that an FLT flare at 2hrs of pemetrexed therapy of similar magnitude to that which was observed in preclinical *in vivo* studies. This clinical trial is still open and recruiting patients. We anticipate that this data will ultimately be published and presented at a national meeting.

The preclinical and clinical trial data has been presented at Grand Rounds in the Department of Radiology and, at the national level, the data has been presented at the ECOG-ACRIN Thoracic Core committee and the AACR 2016 and AACR 2017 international annual meetings. In addition, the data from this project has resulted in two papers published as original research in the journal Oncotarget. One was published in September 2016 (PMID: 27655645) and the other is in press. Please see the **Appendix** for copies of these papers.

What opportunities for training and professional development has the project provided?

During the 2 year reporting period, Dr. Katz met routinely with her mentors, Drs. Schnall, Mankoff, Albelda and Langer as outlined in the Statement of Work. In addition, Dr. Katz has participated in the relevant meetings and seminars are outlined in the Statement of Work. These interactions have assisted in supporting the progress of both the preclinical aim and clinical trial.

In addition, Dr. Katz, under the mentorship of Drs. Schnall and Mankoff, has engaged in the ECOG-ACRIN organization integrated into the Thoracic Core Committee. Dr. Katz attended the Fall 2014, Spring 2015, Fall 2015, Spring 2016 and Fall 2016 ECOG-ACRIN working group meetings. Networking during these meetings has led to Dr. Katz giving 3 research talks to the ECOG-ACRIN Thoracic core committee resulting in new collaborations.

In addition to Dr. Katz, Ms. Urooj Khalid, a University of Pennsylvania undergraduate student, and Mr. Ian Berger, a University of Pennsylvania Perelman School of Medicine student were engaged in the clinical trial proposed in Specific Aim #2, Under the mentorship of Dr. Katz, Ms. Khalid and Mr. Berger have had met weekly with Dr. Katz and have learned the basics of clinical trial design, have had hands on experience with patient recruitment and have been actively involved in day-to-day problem solving issues related to the clinical trial. Both of these students have presented their work at meetings under the mentorship of Dr. Katz.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

The work accomplished on the pre-clinical aim succeeded in determining the optimal timing to measure the FLT-PET "flare". This information was integrated into the proposed exploratory clinical trial which is now in progress. Ultimately, if the use of the FLT-PET "flare" technique is successful in clinical trials, the information on optimal timing will be translatable to a multicenter clinical trial.

What was the impact on other disciplines?

If the use of the FLT-PET "flare" technique is successful in clinical trials, the information on optimal timing may ultimately be translatable to a clinical use in oncology and other disease entities that employ use of thymidylate synthase (TS) inhibition as a drug therapy.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

The clinical trial protocol design was modified to allow for imaging of the FLT "flare" response to pemetrexed during the 2nd infusion of 1st line therapy. This was done to improve patient recruitment to the trial. These changes were submitted and approved by the PENN IRB and submitted to the DOD HRPO.

Actual or anticipated problems or delays and actions or plans to resolve them

Recruitment to the clinical trial has been very slow and multiple steps were taken to improve recruitment including regular communication with the oncologists, volunteer staffing in the oncology clinics, initial increase in reimbursement to the patients and modification of the protocol to allow for imaging during the 2nd infusion cycle. In addition, the PI has routinely held weekly research meeting with the study staff to discuss recruitment. Despite these efforts, this clinical trial has not recruited well. This is felt to be on the basis of multiple factors the most consequential of which is shift of the field to immunotherapy for treatment of lung cancer. Additional challenges have included difficulty engaging this population of terminal cancer patients near the time of initial diagnosis of their cancer for a clinical trial that offers no therapeutic benefit and therapeutic clinical trials competing for the same patient population.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

There were no significant deviations or unexpected outcomes for the clinical trial protocol. Several minor modifications of the clinical trial were made to allow for changes in study staffing, increase in reimbursement to patients and change of the timing of the FLT flare imaging to 2nd infusion of the 1st line therapy (rather than the initial infusion as initially proposed).

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations
Report only the major publication(s) resulting from the work under this award.

Journal publications.

1) Xiao Chen, Yizeng Yang, Ian Berger, Urooj Khalid, Akash Patel, Jenny Cai, Michael D. Farwell, Corey Langer, Charu Aggarwal, Steven M. Albelda and Sharyn I. Katz. "Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small cell lung cancer with FLT-PET imaging." Oncotarget. 2016 Sep 16. (E published)
2) Xiao Chen, Yizeng Yang and Sharyn Katz. "Early detection of thymidylate synthase resistance in non-small cell lung cancer with FLT-PET imaging." (Oncotarget, Epub Jul 31, 2017)

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

Presentation of preclinical work at the annual meeting of the AACR 2016 in New Orleans, LA and AACR 2017 in Washington, DC (international meetings).

Presentation of the preclinical and clinical work at the ECOG-ACRIN Thoracic core committee (national meeting).

Presentation of work at the UPENN Department of Radiology research symposium 2016 (national)

Grand Rounds talk, Department of Radiology, UPENN. (local meeting)

- **Website(s) or other Internet site(s)**

Below is the URL for the published article that derived from this grant funding:

http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path%5B%5D=12085

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

> *Nothing to Report.*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sharyn Katz,	XiaoChen	Urooj Khalid	Ian Berger	Jenny Cai
Project Role:	Principal Investigator	Research Assistant	Student Research Assistant	Student Research Assistant	Clinical Research Assistant
Researcher Identifier (e.g. ORCID ID):					
Nearest person month worked:	9	8	2	2	2
Contribution to Project:	Dr. Katz is the project PI and leads the preclinical aim and exploratory clinical trial	Dr. Chen provides technical support in the laboratory for the preclinical aims of this project.	Ms. Khalid is an Undergraduate student at the University of Pennsylvania and assists in recruitment of potential patients to the project clinical trial.	Mr. Berger is a medical student at the University of Pennsylvania Perelman School of Medicine and assists in recruitment of potential patients to the project clinical trial.	Ms. Cai is a clinical research assistant at the Hospital of the Univ. of Pennsylvania and assists in patient recruitment, accompanying the patient on the day of the FLT scans, and maintenance of the regulatory binder.
Funding Support:	DOD LRCP CDA,American Cancer Society-Investigator Research Grant	Self-funded through funds from her home	Funded through the PI's, University of Pennsylvania University	Not funded (volunteer)	Supported through funds through the Dept. of Radiology, Hospital of

	(ACS-IRG), UPENN University Research Fund (URF)	university in China	Research Fund (URF) Award		the Univ. of Pennsylvania

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report."

What other organizations were involved as partners?

University of Pennsylvania, Department of Radiology: coverage of costs of FLT-PET scans and preclinical work

Investigator Research Grant (ACS-IRG): coverage of cost of clinical FLT-PET scans

University of Pennsylvania University Research Fund (URF) Award: coverage of cost of clinical FLT-PET scans

8. **SPECIAL REPORTING REQUIREMENTS**

   COLLABORATIVE AWARDS: N/A

   QUAD CHARTS: N/A

9. **APPENDICES:** (Oncotarget journal publications and AACR abstracts)

Appendix 1

Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small cell lung cancer with FLT-PET imaging

Xiao Chen[1,2], Yizeng Yang[3], Ian Berger[1], Urooj Khalid[1], Akash Patel[1], Jenny Cai[1], Michael D. Farwell[1], Corey Langer[3], Charu Aggarwal[3], Steven M. Albelda[3] and Sharyn I. Katz[1]

[1] Department of Radiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

[2] Department of Radiology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China

[3] Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

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ABSTRACT

Inhibition of thymidylate synthase (TS) results in a transient "flare" in DNA thymidine salvage pathway activity measurable with FLT ($[^{18}F]$ thymidine)-positron emission tomography (PET). Here we characterize this imaging strategy for potential clinical translation in non-small cell lung cancer (NSCLC). Since pemetrexed acts by inhibiting TS, we defined the kinetics of increases in thymidine salvage pathway mediated by TS inhibition following treatment with pemetrexed *in vitro*. Next, using a mouse model of NSCLC, we validated the kinetics of the pemetrexed-mediated "flare" in thymidine salvage pathway activity *in vivo* using FLT-PET imaging. Finally, we translated our findings into a proof-of-principle clinical trial of FLT-PET in a human NSCLC patient. In NSCLC cells *in vitro*, we identified a burst in pemetrexed-mediated thymidine salvage pathway activity, assessed by ^{3}H-thymidine assays, thymidine kinase 1 (TK1) expression, and equilibrative nucleoside transporter 1 (ENT1) mobilization to the cell membrane, that peaked at 2hrs. This 2hr time-point was also optimal for FLT-PET imaging of pemetrexed-mediated TS inhibition in murine xenograft tumors and was demonstrated to be feasible in a NSCLC patient. FLT-PET imaging of pemetrexed-induced TS inhibition is optimal at 2hrs from therapy start; this timing is feasible in human clinical trials.

INTRODUCTION

Successful inhibition of thymidylate synthase (TS), a critical enzyme in the *de novo* thymidine synthesis pathway, is the key target of chemotherapeutics such as 5-fluorouracil (5-FU), pemetrexed, and capecitabine. Once TS is blocked, a rapid compensatory increase in the thymidine salvage pathway occurs resulting in a rapid uptake of extracellular thymidine. This burst or "flare" in uptake can be visualized using ^{18}F-thymidine (FLT), an analogue of thymidine and a PET (positron emission

tomography) radiotracer. FLT was first described as an imaging biomarker of thymidine salvage activity by Shields and Grierson *et al* in 1998 [1] and is a validated surrogate marker of proliferation in lung cancer [1-4]. In the cell, FLT becomes mono-phosphorylated and trapped by the key thymidine salvage pathway enzyme thymidine kinase 1 (TK1); thus tumors become more FLT-avid as thymidine salvage pathway activity increases. As such, this drug-induced salvage pathway "flare" effect provides an imaging opportunity to determine successful TS inhibition in the tumor within hours of starting therapy.

The TS-inhibition induced FLT "flare" effect appears to be mediated primarily though one or both of two mechanisms. The first is an increase in TK1 function, the rate-limiting step of the thymidine salvage pathway. This may occur either through an increase in TK1 activity [5, 6], which is modulated by its physical state [7], or protein expression of TK1[8] [9]; both of these effects are carefully modulated throughout the cell cycle [5, 10, 11]. This boost in TK1 function serves to compensate for the inhibition of the *de novo* synthesis pathway allowing continued supply of thymidine for cellular division. Increased cell surface density of equilibrative nucleoside transporter 1 (ENT1) may also contribute to the FLT "flare". This may occur either from ENT1 mobilization to the cell surface [12] or an increases in ENT1 expression [6]. ENT1 transport is regulated by the cell cycle and is the dominant mechanism of increased FLT entry for proliferating cells [13-15]. In some studies ENT1 has been shown to rapidly mobilized to the cell surface within hours of successful TS-inhibition [12, 16] while others have failed to observe this shift in ENT1 distribution [5].

It is still uncertain whether this FLT "flare" imaging technique can be a reliable predictor of tumor response to therapy. A recent clinical pilot study of FLT "flare" as a measure of response to therapy with pemetrexed-based therapy in NSCLC showed no association between the presence of the FLT "flare" and clinical outcome [17]. Though this study had a small heterogenous population of patients, it does raise the need for further pre-clinical modeling to fully characterize this imaging strategy prior to clinical translation. In order to study the predictive value of this technique, it is first critical to determine the optimal timing of measurement of the "flare". The TS-inhibitor mediated thymidine salvage pathway "flare" is a transient metabolic phenomenon which dissipates within hours and there has been variability in the reported timing of measurement of this effect from 1-48 hrs following exposure to therapy [8, 16-21]. This variability is likely due to differing mechanisms of the "flare" depending on cancer type and specific TS inhibitor therapy. We focus here on pemetrexed, a TS-inhibitor commonly used in 1st line therapy for non-small cell lung cancer.

In this study, we define the kinetics of the pemetrexed-induced FLT "flare" in order to determine the optimal timing of FLT imaging for further preclinical study and ultimately translation to the clinic. Furthermore, we elucidate the mechanism of FLT "flare" following pemetrexed-induced inhibition and characterize the potential impact of concurrent therapy with a platin drug on "flare" kinetics. This is important since pemetrexed regimens typically include a DNA-damaging platin agent such as carboplatin or cisplatin. Finally, we conduct a pilot of FLT-PET imaging of pemetrexed-induced TS inhibition in a patient with NSCLC to validate the feasibility of this imaging technique at the determined optimal time point.

RESULTS

Pemetrexed-induced TS inhibition results in a "flare" in thymidine salvage pathway activity peaking at 2 hours *in vitro* which is partially blocked by ENT1 inhibition

Initially, we sought to define the kinetics the TS inhibition-induced "flare" of the thymidine salvage pathway in NSCLC cells *in vitro*. H460 and H1299 NSCLC lines, both pemetrexed sensitive (Supplemental data Figure S2), demonstrated a significant transient compensatory "burst" in thymidine salvage pathway activity peaking at 2 hours as measured on ^{3}H-thymidine assays (Figure 1) relative to untreated controls. The average magnitude of the thymidine salvage pathway "flare" at 2 hours was 38% above baseline in H460 and 35% over baseline in H1299. The addition of cisplatin to pemetrexed treatment of these cells did not impact the amplitude or timing of the thymidine salvage pathway "flare" effect (H460: $p = 0.32$; H1299: $p = 0.12$).

In order to determine if intact ENT1 function was required for this pemetrexed-induced DNA salvage pathway "flare", pemetrexed-induced changes in DNA salvage pathway activity was also examined in the presence of ENT1 inhibition. When pre-treated with ENT1 inhibitor NBMPR (Figure 1), the pemetrexed-induced "flare" in DNA salvage pathway was markedly diminished in both the H460 and H1299 NSCLC cell lines. In the H460 and H1299 cell lines, the magnitude of the pemetrexed-induced "flare" at 2hrs was 3.2% and 7.3% over the baseline respectively. With the addition of cisplatin to pemetrexed, there was no significant change in the blunting of the DNA salvage pathway flare observed in the presence of ENT1 inhibition. For H460 and H1299 cells treated with the combination of pemetrexed and cisplatin, the magnitude of the pemetrexed-induced DNA salvage pathway flare at 2 hours was 2.8% and 11.87% over baseline respectively. The addition of cisplatin to pemetrexed treatment of these cells did not impact the amplitude or timing of the thymidine salvage pathway effect: H460: $p = 0.96$; H1299: $p = 0.34$).

By 24 hours of therapy, both cells treated with pemetrexed and cells treated with a combination therapy of pemetrexed and cisplatin exhibited a significant suppression of proliferation relative to untreated controls. For cells treated with pemetrexed alone, intracellular ^{3}H-thymidine accumulation was decreased by 36% and 19% at 24 hours of exposure relative to baseline for H460 and H1299 respectively. At 24 hours of exposure to combination therapy with pemetrexed and cisplatin, intracellular ^{3}H-thymidine accumulation decreased by 75% and 42% relative to baseline for H460 and H1299 respectively. Significantly greater suppression of

proliferation was exhibited by the combination therapy with cisplatin and pemetrexed versus pemetrexed alone (H460: $p = 0.007$; H1299: $p = 0.016$). Thus, for pemetrexed alone and in combination with cisplatin, the "flare" effect was optimal *in vitro* at 2 hours after starting therapy. These findings were validated in 6 additional human NSCLC cell lines demonstrated to be sensitive to pemetrexed and cisplatin (Supplemental data Figure S2 and S3).

Pemetrexed-induced thymidine salvage pathway "flare" is a result of a combination of redistribution of ENT1 receptors and activation of TK1 to the tetramer state

In order to gain insights into the mechanism for the pemetrexed-induced thymidine salvage pathway "flare", the protein expression of several key components of the thymidine salvage pathway were examined following exposure to pemetrexed or pemetrexed plus cisplatin.

TK1, rate-limiting enzyme of the thymidine salvage pathway, exists in a modestly active monomer, moderately active dimer and highly active tetramer state. Treatment of both H460 and H1299 cells revealed a shift in TK1 protein to the tetramer state following 2 hours of exposure to pemetrexed therapy, with an increase of TK1 in the tetramer state of 10 fold ($p < 0.0001$) and 1.6 fold ($p = 0.011$), respectively, and corresponding decreases in the monomer and dimer states of TK1 (Figure 2). These data are consistent with the timing of the pemetrexed-induced "flare" in thymidine salvage pathway activity observed at 2 hours using ^{3}H-thymidine assays (Figure 1). Similar to data from the ^{3}H-thymidine assay data, the induction of this tetramer state was unaffected by exposure to cisplatin (H460: $p = 0.48$; H1299: $p = 0.70$).

Levels of total TS and ENT1 protein expression were not significantly changed during the 1st 8 hours of treatment. However, by 16-24 hours of exposure of cells to pemetrexed plus cisplatin, both cell lines exhibited a gradual decrease in protein expression of TK1, TS and ENT1 in keeping with successful cell cycle arrest. At 24
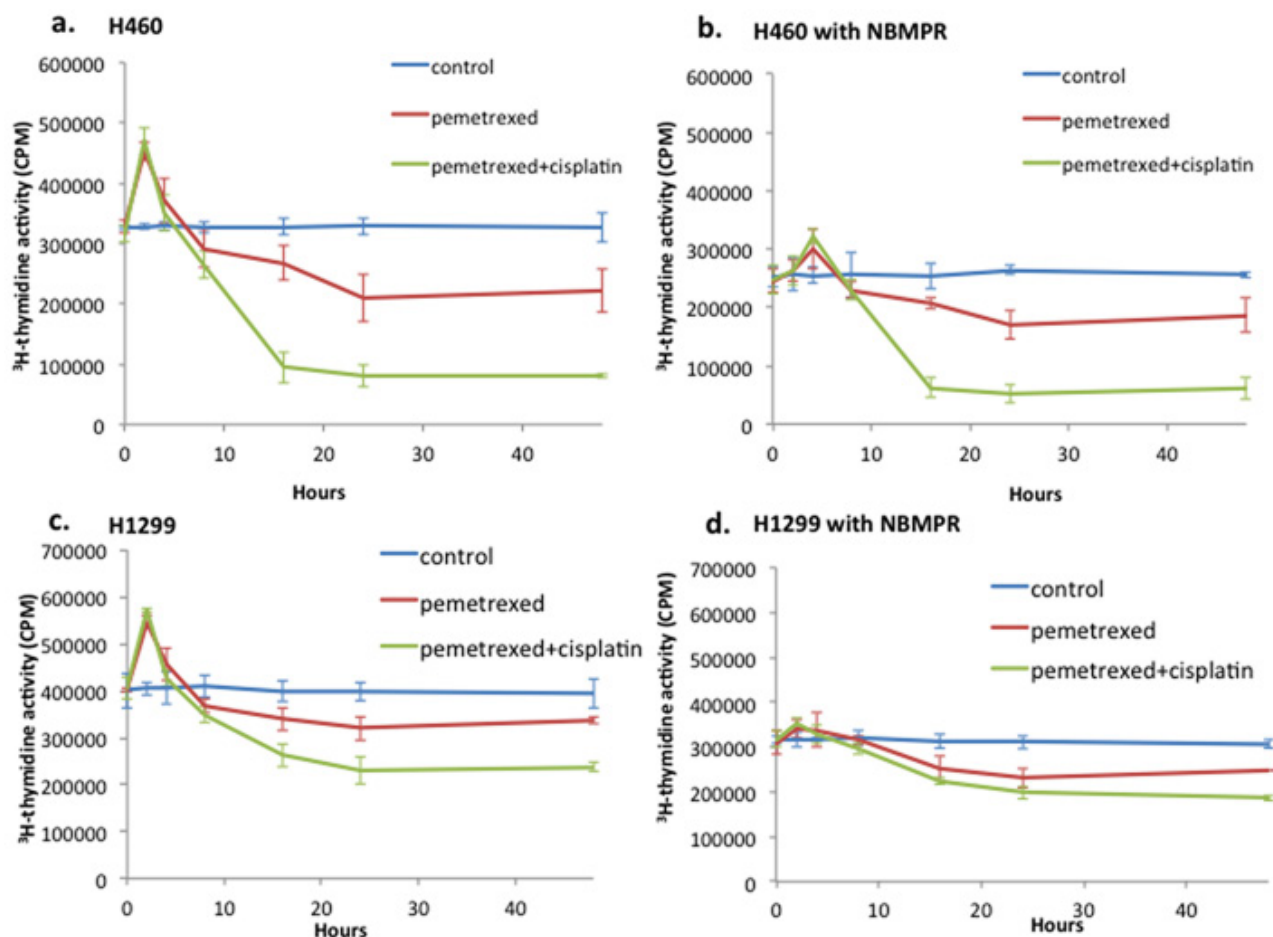

Figure 1: Pemetrexed-induced TS inhibition results in a "flare" of the thymidine salvage pathway activity. ^{3}H-thymidine assay was performed on PEM-sensitive NSCLC H460 in untreated control (culture medium only), pemetrexed (100nM) and combination therapy with pemetrexed (100nM) plus cisplatin (10mM). A "flare" of thymidine salvage pathway activity peaked at 2 hours of pemetrexed therapy exposure in both the H460 **a.** and H1299 **c.** NSCLC cell lines. This "flare" in thymidine salvage pathway activity was blunted by pretreatment of cell cultures with ENT1 inhibitor NBMPR in both H460 **b.** and H1299 **d.** NSCLC cell lines.

hours, there was widespread decrease in expression in the measured proteins relative to baseline. TK1 (total protein) was decreased by 36% ($p = 0.0092$) and 35% ($p = 0.007$), TS decreased by 38% ($p = 0.04$) and 8.67% ($p = 0.64$), and ENT1 decreased by 17% ($p = 0.19$) and 84% ($p = 0.0005$) for H460 and H1299 respectively.

In addition to examination of protein expression of key elements of the thymidine salvage pathway, we also examined subcellular localization of ENT1. By immunofloresence microscopy, we detected a transient but significant shift in subcellular localization of ENT1 protein from the peri-nuclear location to the cell surface following treatment with pemetrexed. Cell-surface localization of ENT1 peaked at 2 hours of exposure to pemetrexed with a 3.9 fold ($p < 0.0001$) and 4.2 fold ($p < 0.0001$) increase for H460 and H1299 respectively. This peak was followed by a steady shift back to peri-nuclear localization with a return to near baseline distribution by 24 hours (Figure 3).

In vivo modeling validates *in vitro* kinetics of pemetrexed-induced "flare" in thymidine salvage pathway activity

Prior to conducting a study of FLT flare kinetics, we confirmed efficacy of combined therapy with pemetrexed and cisplatin *in vivo*. A pilot study of 12 H460 tumor-bearing xenografts, 4 control (vehicle only) and 8 treated (combination of pemetrexed and cisplatin), revealed tumor growth inhibition of 88% in those treated with pemetrexed and cisplatin relative to vehicle only controls (Supplemental data Figure S1). Consequently, *in vivo* modeling of 1st line therapy of NSCLC xenografts with combined pemetrexed and cisplatin therapy revealed a peak in tumor avidity for FLT at 2 hours of exposure to therapy (Figure 4). At 2 hours of therapy, tumor FLT_{MAX} increased 47.5% ($\pm12.0\%$, $p = 0.008$) relative to baseline in the xenografts treated with cisplatin and pemetrexed but remained unchanged in vehicle treated controls ($p = 0.37$). After this post-therapeutic peak or "flare" in tumor avidity for FLT at 2 hours, tumor avidity for FLT decreased over the course of 24 hours. By 24 hours following start of combination therapy with pemetrexed plus cisplatin, tumor avidity for FLT began to fall below baseline, albeit not significantly, with a mean tumor FLT_{MAX} of -6.0% ($\pm10.5\%$, $p = 0.35$). This is likely due to the impact of successful therapy on proliferation, which occurs in a similar timeframe to the *in vitro* studies of thymidine salvage pathway activity and protein expression (Figure 1 and 2).

Human FLT-PET imaging of pemetrexed-induced "flare" at 2 hours of therapy is feasible

We next sought to extend our findings to human lung cancer through a proof-of-concept clinical trial of
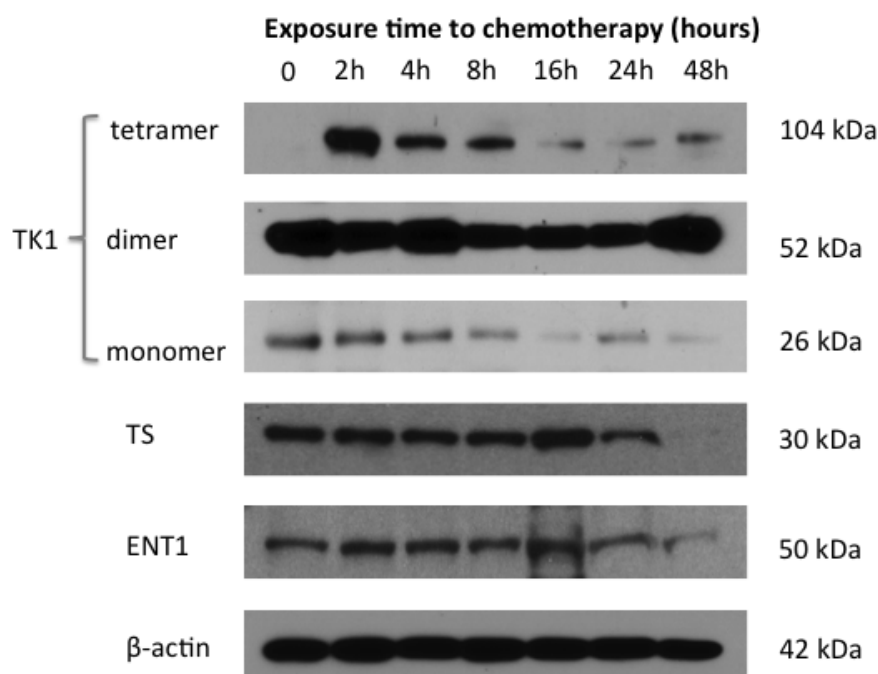

Figure 2: Induction of tetramer state of TK1 corresponds temporally with pemetrexed-induced thymidine salvage pathway "flare." A time course of exposure of PEM-sensitive NSCLC cell line H460 to combination therapy with PEM/cisplatin *in vitro* revealed induction of highly activated tetramer TK1 state corresponding temporally to the FLT "flare" observed at 2 hours. ENT1 and TS protein levels remain unchanged in the "flare" period. All protein levels slowly decreased beyond 8 hours due to successful cell cycle inhibition by PEM/cisplatin therapy.

FLT-PET/CT as a measure of TS inhibition in patients with advanced NSCLC. Baseline FLT-PET performed three days prior to start of therapy revealed a FLT-avid (SUV_{MAX} of 5.4) solid 6 cm mass in the left upper lobe in keeping with the known NSCLC. A repeat FLT-PET/CT scan performed at 2 hours following the first infusion of combined therapy with pemetrexed and carboplatin revealed a post-therapeutic increase in tumor avidity for FLT with a 44% increase in SUVmax (SUV_{MAX} of 7.8) relative to baseline (Figure 5). Thus, in a human patient with NSCLC, FLT-PET revealed a "flare" in tumor avidity for FLT at 2 hours following intravenous infusion with pemetrexed and carboplatin; this study demonstrates the feasibility of translating this technique, using a 2 hour time point, to the clinic. Nonetheless, more patients will need to be studied to validate the predictive value of this post-therapeutic FLT "flare" for lung cancer patients treated with 1st line pemetrexed-based therapies.

DISCUSSION

In this study we determine the optimal timing for imaging the pemetrexed-induced thymidine salvage pathway "flare" in NSCLC. To our knowledge this is the first detailed study of timing and mechanism of pemetrexed-induced DNA salvage pathway "flare" in NSCLC. *In vitro* and *in vivo* data in this study consistently show a transient peak in thymidine salvage pathway "flare" activity at 2 hours of exposure to pemetrexed. This timing of the thymidine salvage pathway "flare" is in the range of that reported in the literature for other TS inhibitors but earlier than the time points used in other clinical trials of TS inhibitor induced FLT "flare" [17, 18]. By optimizing the timing of the FLT "flare" in pemetrexed therapy, future studies will be positioned to determine the predictive value of this imaging strategy in this therapeutic setting. Moreover, to our knowledge, this is the first study to determine that concurrent treatment with cisplatin does not alter the timing or magnitude of the TS-inhibition mediated salvage pathway "flare." This is critical to the utility of this technique for clinical translation of this imaging strategy since pemetrexed is frequently used in combination with a platin-based therapeutic for oncologic management.

Tumor avidity for FLT is well correlated with TK1 expression [23] and activity [10], the rate-limiting step in the thymidine salvage pathway [23], and with the cell surface expression of ENT1 [13, 14]. Analysis of protein
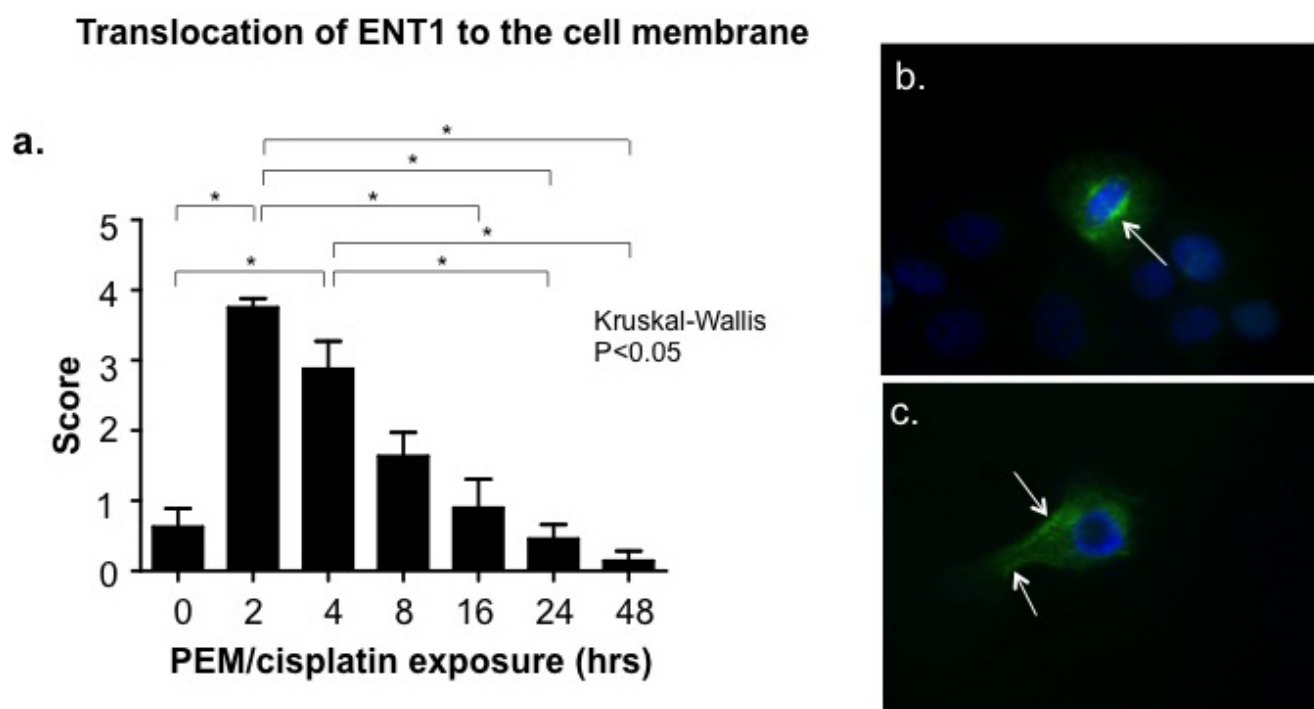

Figure 3: Translocation of ENT1 to the cell surface corresponds temporally with the pemetrexed-induced thymidine salvage pathway "flare." Immunofloresence microscopy utilizing a time course of exposure of the pemetrexed (PEM)-sensitive NSCLC cell line H460 to combination therapy with PEM(100nM)/cisplatin(10 μM) *in vitro* revealed translocation of ENT1 to the cell surface from the peri-nuclear cytoplasm maximal at 2 hours of exposure to PEM or PEM/cisplatin corresponding to the timing of the FLT "flare". **a.** Cells were scored on a scale 0 (no ENT1 translocation) to 5 (high ENT1 translocation to the cell surface) Microscopy demonstrated NSCLC staining for ENT1 (Green stain) and nuclear membrane (blue stain) following 2 hours of exposure to **b.** culture medium control **c.** or combination of pemetrexed and cisplatin. Arrows depict concentration of ENT1 staining.

expression in this study suggests that the pemetrexed-induced salvage "flare" effect is likely attributable, at least partially, to an increase in TK1 activity via a shift in enzyme state to highly active tetramer. Our data also indicate that ENT1 contributes to the pemetrexed-induced "flare" in DNA salvage pathway activity and that transient localization of ENT1 to the cell surface likely contributes to this thymidine salvage pathway "flare"

effect. There is support in the literature for both increases in TK1 activity[5, 6, 8] and redistribution of ENT1 to the cell surface[12, 16] as potential mechanisms for the TS-inhibition mediated thymidine salvage pathway "flare" effect. The ENT1 transporters may also contribute to the subsequent decrease in tumor avidity for FLT following the "flare" as they have been demonstrated to contribute to nucleoside/nucleotide efflux from the cell[24] and to
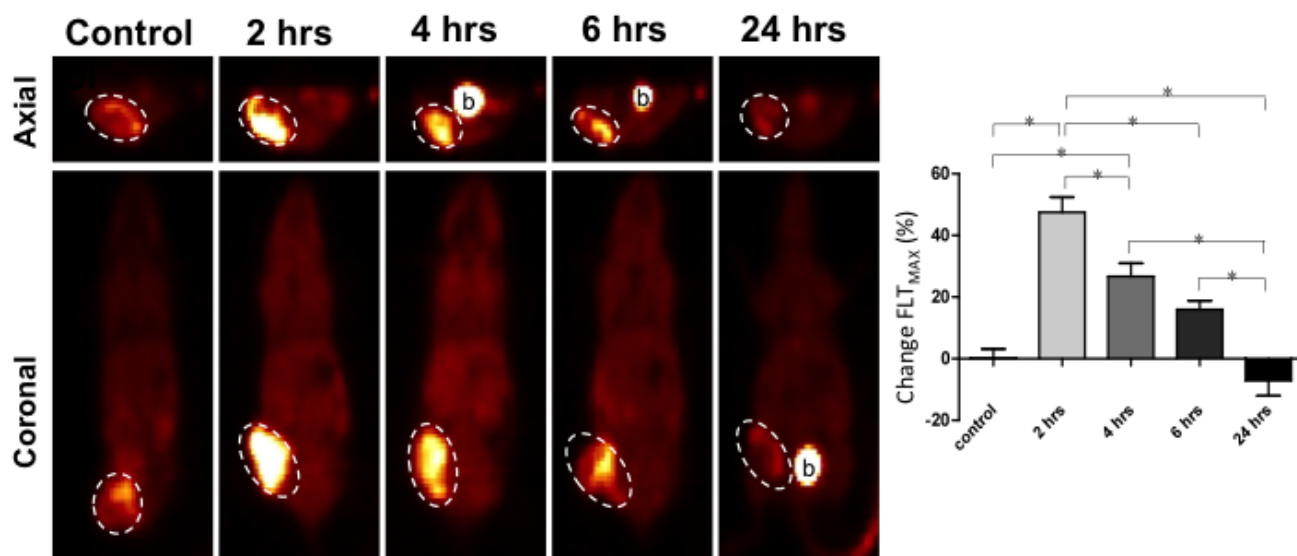

Figure 4: FLT-PET imaging of pemetrexed-induced TS inhibition demonstrates a FLT "flare" peaking at 2 hours in a preclinical model of NSCLC. Human NSCLC tumor-bearing murine xenografts were treated with a combination of pemetrexed (PEM) and cisplatin in order to model 1st line therapy. FLT-PET was performed at baseline and at multiple time points following therapy start. Tumor avidity for FLT was observed to peak at 2 hours following PEM-based therapy. By 24 hours of therapy, tumors began to demonstrate inhibition of proliferation. (b.) Excreted radiotracer within the bladder.
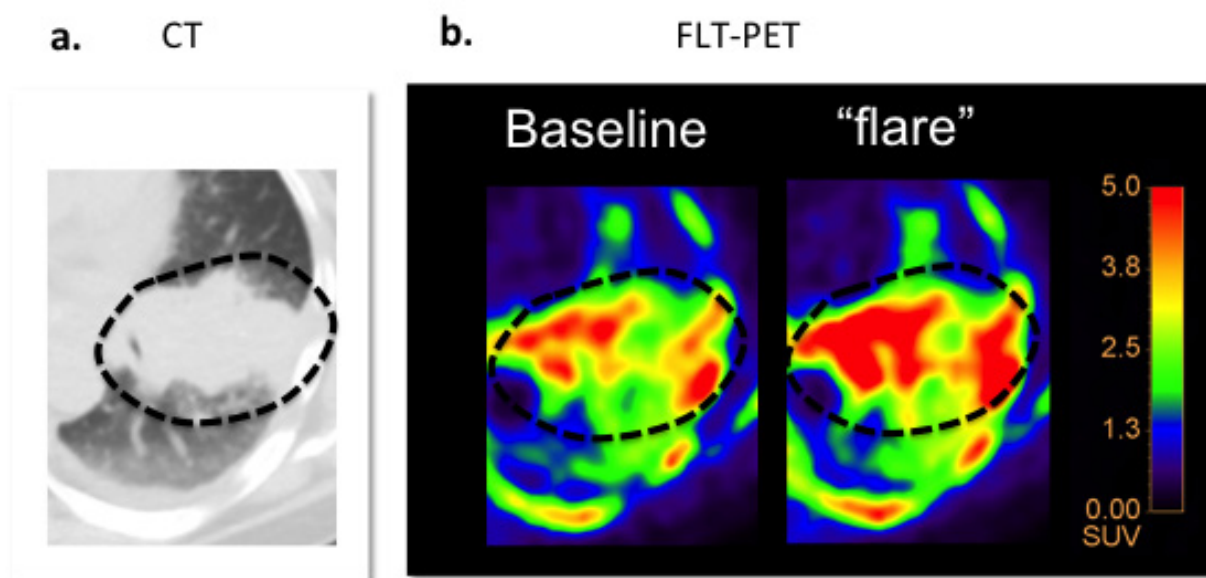

Figure 5: FLT "flare" in response to PEM-based therapy in a human patient. These images are from a 63 year-old male with NSCLC participating in our exploratory clinical trial of FLT-PET "flare". **a.** Baseline CT revealed a 6 cm mass in the left upper lobe. **b.** Baseline FLT-PET revealed mild avidity 3 days pre-therapy. **c.** FLT-PET "flare" imaging performed after 2 hours following administration of combination therapy with PEM and carboplatin revealed a burst in tumor avidity in keeping with the FLT "flare."

be decreased in expression as cells exit active cell cycling [13] in response to therapy.

Our data also demonstrate that the pemetrexed-induced FLT "flare" is not mediated by changes in TK1 or ENT1 protein expression. This is plausible since changes in protein expression are unlikely to occur in a 2 hour time interval. Lee *et al* described a bimodal FLT "flare" occurring at 2 hours and 24 hours in most cell lines treated with 5-FU[8]. This later 24-48 hour peak in salvage pathway "flare" may be due to increased expression of TK1 [8, 16] and/or ENT1 [6], which is observed following mono-therapy with 5-FU [6, 8] or BGC 945 [16]. We hypothesize that the nature of the therapeutics, such as the choice of TS inhibitor and use of mono-therapy versus combination therapy, may impact contributions of available salvage pathway compensatory mechanisms resulting in differing FLT "flare" kinetics. As such, we posit that our use of concurrent therapy with a DNA damaging platin agent prevents compensation of the DNA salvage pathway through increases in protein expression, seen in some models at 24-48 hours, due to overall suppression of protein expression. Of note, while ENT1 is the dominant transporter for FLT, in this study we did not evaluate the potential contributions of other cell surface transporters such as the concentrative transporters, which are known to contribute to FLT metabolism [14, 24] and may also play a role in the dynamics of pemetrexed-induced FLT "flare".

Our study results support previously published data that the TS-inhibition induced "flare" in thymidine salvage pathway activity rapidly dissipates. Using pemetrexed therapy, the "flare" effect was dissipated by 24 hours both *in vitro* and *in vivo*. By 24 hours, the effects of the chemotherapeutics have begun to impair tumor proliferation with resulting suppression of thymidine salvage pathway activity and protein synthesis. These findings are compatible with the findings of Barthel *et al* who demonstrated that tumor avidity for FLT is significantly decreased relative to baseline by 48 hours of exposure to 5-FU [25]. This anti-proliferative effect is greater with combined therapy of pemetrexed and cisplatin compared to pemetrexed alone, in keeping with the fact that these cell lines are sensitive to both pemetrexed and cisplatin. In light of these data we propose that an imaging strategy designed to specifically detect the success of pemetrexed-induced TS inhibition should measure thymidine salvage pathway at 2 hours. In contrast, imaging designed to evaluate the impact of pemetrexed-induced effects on proliferation should measure thymidine salvage pathway activity after 24 hours of therapy. Additional preclinical modeling will be necessary, using dynamic PET imaging, to determine the potential impact of pemetrexed-based therapeutics on the relative blood bioavailability of FLT metabolites as this can impact quantitation of FLT and will be the subject of future studies.

Finally, our pilot of the FLT "flare" imaging strategy in a patient with NSCLC suggests that FLT-PET imaging

at the 2 hour window following infusion with pemetrexed-based therapy is feasible in the clinic and a clear "flare" can be imaged at this time point. Further study will be necessary to determine the predictive value of this imaging strategy. It is plausible that detection of response to pemetrexed-based therapy at this early time point, months before what is currently possible by conventional assessments with computed tomography (CT), will have a benefit to the patient in terms of time saved during which tumors can grow and patients are exposed to unwarranted therapeutic toxicities. In sum, our data provide a rationale for using the 2 hour time point for studies of pemetrexed-induced FLT-PET "flare" when modeling 1st-line therapy in NSCLC. Future studies are needed to determine the predictive value of this imaging technique as well as the potential for benefit to the patient by clinical translation.

MATERIALS AND METHODS

Chemotherapeutics and imaging radiopharmaceuticals

For *in vitro* studies, pemetrexed (Santa Cruz Biotechnology, Dallas, Texas) and cisplatin (Sigma-Aldrich Corp., St. Louis, MO) were provided in solid form, dissolved in water and stored at -20°C as a 0.2 mM and 1 mM stock, respectively. For *in vivo* use, both human and murine, pemetrexed (ALIMTA; Eli Lilly and Company, Indianapolis, IN) and cisplatin (Teva Pharmaceuticals, Petach Tikva Israel) were provided freshly prepared as a 1mg/ml sterile saline solution by the Abramson Cancer Center Pharmacy. For *in vivo* murine studies, chemotherapeutics were stored at 4°C. [18F]FLT was produced on site in the University of Pennsylvania PET Center Cyclotron facility. [18F]FLT average specific activity was 5.32 +/- 2.14 Ci/umol, and radiochemical purity >99%.

Cell lines and culture

All human non-small cell lung cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in RPMI medium containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator in 5% CO2 at 37°C. Passage of cell lines was performed at 1:3 dilution after detachment using sterile 0.05% trypsin-EDTA solution.

IC50 calculations

Cultured cell lines were harvested and seeded into a 24-well plate (2 X10^4 cells per well) in RPMI1640 culture

medium and incubated for 24 hours at 37°C in a 5% CO2 incubator. The culture medium was then replaced with 100 uL of fresh medium containing varying concentrations of pemetrexed (0, 0.01, 0.1, 1, 10, 100μM) and incubated for 72 hours at 37°C in a 5% CO2 incubator. The IC50 assay was performed then performed using the MTT Cell Growth Assay Kit (Sigma-Aldrich, St. Louis, MO). Absorbance of the converted dye was measured using a Beckman DU-600 Spectrophotometer (Beckman Coulter Life Sciences, Indianapolis, IN) and data analyzed using the statistical software SPSS 19.0 (IBM, Chicago, USA).

Mouse tumor xenograft modeling

Prior to *in vivo* animal modeling, approval was obtained by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Human tumor-bearing murine xenografts were then created using two month-old female *nu/nu* mice (Crl: NUFoxnlnu, Charles River Laboratory, Wilmington, MA). Each mouse was injected subcutaneously in the flank with a suspension of H460 cells (1×10^7) in sterile, endotoxin-free 50% Matrigel Matrix (Corning Inc., Corning, NY). When tumors reached a mean volume of approximately 200 mm^3 (volume = $\pi/6 \times$ length $\times$ width $\times$ height), animals were randomized into treatment groups.

In vitro analysis of thymidine salvage pathway activity

³H-thymidine assays

[³H]-thymidine (Perkin Elmer NET355001MC, PerkinElmer, Waltham, MA) was utilized for *in vitro* assessment of therapy-induced changes in thymidine salvage pathway activity in cultured human NSCLC cells. [³H]-thymidine specific activity was >10Ci(370GBq)/mmol and radiochemical purity >97%. H460 and H1299 cells were seeded (1×10^6/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 hours in 5% CO$_2$ at 37°C. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM) and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48 hours. Drug-containing medium was then removed, and the cells were then washed and pulsed with 5 μCi [³H]-thymidine/well for 1 h. The cells were then washed and scraped into plastic vials. Scintillant (10 ml; Research Products International Corp., Mount Prospect, IL) was added to each vial and the radioactivity was counted on a scintillation counter (Beckman Coulter LS6500, Beckman Coulter Life Sciences, Indianapolis,

IN). For ENT1 inhibition, cells were incubated with NBMPR (Nitrobenzylthioinosine) for 15min (100uM; Sigma-Aldrich, St. Louis, MO) prior to labeling with [³H]-thymidine.

Immunoblotting

Therapy-induced changes in the protein expression of key components of the thymidine salvage pathway were assessed using Western blot analysis. H460 and H1299 cells were seeded (1×10^6 cells/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 h in 5% CO$_2$ at 37°C. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM) and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48 hours. Whole cell lysates were then generated using a 1% Nonidet P-40 lysis buffer (Sigma-Aldrich Corp., St. Louis, MO). The suspension was homogenized by passages through a 20-gauge syringe needle and nuclear material removed through centrifugation at 14000 rpm for 15 min at 4°C. Cell lysates were then loaded onto a precast Nupage Bis-Tris Gels (invitrogen, Life Technologies, Corp., Grand Island, NY) for electrophoresis then transferred onto Hybond-P PVDF membranes (Sigma-Aldrich Corp., St. Louis, MO) for Western blot analysis. After blocking membranes with 5% non-fat milk in PBS with 0.1% Tween-20 buffer, PVDF membranes were probed using primary antibodies directed against human TK1 (Cell Signaling Technology, Danvers, MA; 1:5000), human TS (Cell Signaling Technology; 1:4000), human ENT1 (Abcam, Cambridge, MA; 1:200), or human β-actin (Sigma-Aldrich Corp., St. Louis, MO; 1:10000). Membrane were then washed and incubated (1:3000) with species-specific secondary antibodies, either anti-rabbit or anti-mouse, conjugated to horseradish peroxidase (GE Healthcare Life Science; 1:3000) for 1 h, the proteins were detected using the Immobilon ECL system (EMD Millipore, Billerica, MA) and quantified using Image J software available through the National Institutes of Health (https://imagej.nih.gov/ij/index.html).

Immunofluorescence

Therapy-induced changes in the intracellular localization of ENT1 were assessed using immunofluorescence. Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Cells were washed with PBS, permeabilized and blocked with 1% BSA / 10% normal goat serum / 0.3M glycine in 0.1% PBS-Tween solution for 1 h. Cells were then incubated with a primary antibodies directed to human ENT1 (Abcam, Cambridge, MA; 1:100) overnight at 4°C. After incubation with the primary antibody, cells were

washed with PBS and incubated with Cy™2 AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA) with at room temperature for 1 h. DAPI (Thermo Fisher Scientific Inc., Grand Island, NY) was used to stain the cell nuclei at a concentration of 0.5 µg/ml. For negative staining control, primary antibody was omitted during the procedure. Digital images were acquired using a Nikon E600 microscope. The results of the membrane positive immunofluorescence of ENT1 were semiquantitatively assessed and scored as previously described[22]. Scoring criteria were defined as follows: 0 for negative (0% to 5% staining); 1 for weakly positive (5% to 20% staining); 2 for moderately positive (20% to 50% staining); 3 and 4 for strongly positive (50% to 75% and ≥ 75%, respectively).

PET scanning and image analysis

Preclinical PET imaging

Baseline FLT-PET scans were performed on the day prior to therapy. Mice were then treated with either the vehicle control (i.p. sterile PBS) or combination therapy with pemetrexed (i.p. 100 mg/kg) and cisplatin (10 mg/kg). A post-therapy FLT-PET was then performed at varying time points following administration of therapy.

For the assessment tumor response to therapy, tumor-bearing animals were treated for a period of two weeks with 100 mg/kg pemetrexed (i.p. daily; days 1-5 and 8-12) and 10 mg/kg cisplatin (i.p. once weekly). During this treatment period, tumor volumes were estimated by external caliper measurements. After therapy/imaging completion, mice were euthanized with carbon dioxide inhalation.

After anesthesia with inhaled isofluorane in O_2 (3% induction, 1.5% maintenance), mice were injected intravenously with 300-350 µCi of [18F]FLT then allowed to recover from the anesthesia during the 60-min uptake time allowed for radiotracer accumulation. At 60 min post-injection, mice were anesthetized and imaged for a 15-min static acquisition on the small animal PET scanner (A-PET, built in collaboration with Philips Medical Systems) located at the University of Pennsylvania Small Animal Imaging Facility.

Human PET imaging

IRB approval was obtained through our Institutional Review Board for use of FLT-PET/CT under an FDA IND in this clinical trial. An adult patient with unresectable non-small cell lung cancer (NSCLC) receiving therapy with pemetrexed and carboplatin was imaged with FLT-PET within 3 days prior to starting therapy and at 2 hours following the 1st administration of intravenous infusion of the chemotherapeutic regimen. Human FLT-PET/CT was performed on the Philips Ingenuity TF scanner (Philips Medical Systems, at the Perelman Center for Advanced Medicine at the University of Pennsylvania. FLT-PET

static images were obtained after a 60 minute uptake time following intravenous injection of 5mCi of [18F]FLT.

Image analysis

For preclinical PET imaging, after acquisition, the images were reconstructed with the manufacturer software and tumor volumes of [18F] FLT quantitated with the freely available Amide 3D software (LG Software Innovations), which allows for multiplanar analysis of the tumor volume. A region of interest (ROI) was traced around the tumor volume creating a 3D ROI that could be sculpted to the 3D tumor perimeter and visually inspected in the axial, coronal and sagittal planes. Statistical analysis of this 3D tumor uptake volume was then performed using Amide software including ROI volume (mm^3), mean counts/pixel, max PET counts/pixel and standard deviation of tumor counts/pixel. PET values generated by the A-PET machine are absolute pixel counts. In order to control for slight differences in radiotracer administration and *in vivo* blood distribution, absolute tumor counts were normalized to flank muscle (tumor ROI pixel value/thigh muscle ROI pixel value).

For human PET imaging, SUV_{MAX} of the primary NSCLC was measured from axial PET/CT fusion images at baseline and post-therapeutic FLT-PET/CT using Philips IntelliSpace Portal (v5.0.2.40009, Philips Healthcare Nederland B.V., Netherlands) software. Post-therapeutic changes in tumor avidity for FLT-PET were then calculated.

Statistics

Analysis for statistical significance between 2 or more groups was performed using student T tests or ANOVA as appropriate. Kruskal-Wallis was performed to compare scoring of different time points for Immunofloresence of ENT1. P-values greater than 0.05 were considered significant. All analyses were performed with SPSS 19.0 (IBM Corp., Armonk, NY).

CONFLICTS OF INTERESTS

None to disclose

GRANT SUPPORT

Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.
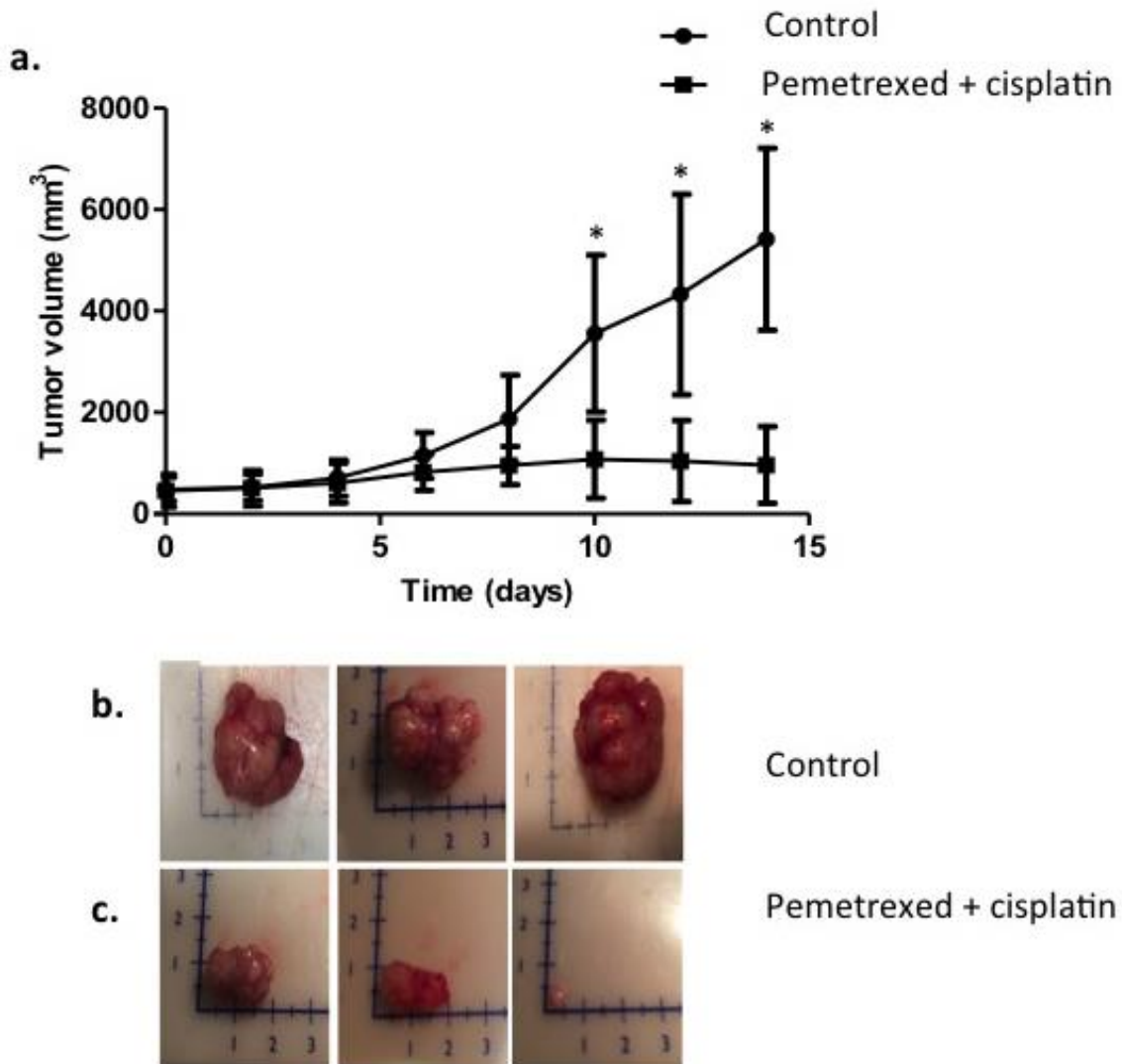
REFERENCES

1. Shields AF, Grierson JR, Dohmen BM, Machulla HJ, Stayanoff JC, Lawhorn-Crews JM, Obradovich JE, Muzik O and Mangner TJ. Imaging proliferation *in vivo* with [F-18]FLT and positron emission tomography. Nat Med. 1998; 4:1334-1336.

2. Muzi M, Vesselle H, Grierson JR, Mankoff DA, Schmidt RA, Peterson L, Wells JM and Krohn KA. Kinetic analysis of 3'-deoxy-3'-fluorothymidine PET studies: validation studies in patients with lung cancer. J Nucl Med. 2005; 46:274-282.

3. Vesselle H, Grierson J, Muzi M, Pugsley JM, Schmidt RA, Rabinowitz P, Peterson LM, Vallieres E and Wood DE. *In vivo* validation of 3'deoxy-3'-[(18)F]fluorothymidine ([(18)F]FLT) as a proliferation imaging tracer in humans: correlation of [(18)F]FLT uptake by positron emission tomography with Ki-67 immunohistochemistry and flow cytometry in human lung tumors. Clin Cancer Res. 2002; 8:3315-3323.

4. Muzi M, Mankoff DA, Grierson JR, Wells JM, Vesselle H and Krohn KA. Kinetic modeling of 3'-deoxy-3'-fluorothymidine in somatic tumors: mathematical studies. J Nucl Med. 2005; 46:371-380.

5. Plotnik DA, McLaughlin LJ, Krohn KA and Schwartz JL. The effects of 5-fluorouracil treatment on 3'-fluoro-3'-deoxythymidine (FLT) transport and metabolism in proliferating and non-proliferating cultures of human tumor cells. Nucl Med Biol. 2012; 39:970-976.

6. Pressacco J, Mitrovski B, Erlichman C and Hedley DW. Effects of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. Cancer Res. 1995; 55:1505-1508.

7. Munch-Petersen B, Cloos L, Jensen HK and Tyrsted G. Human thymidine kinase 1. Regulation in normal and malignant cells. Adv Enzyme Regul. 1995; 35:69-89.

8. Lee SJ, Kim SY, Chung JH, Oh SJ, Ryu JS, Hong YS, Kim TW and Moon DH. Induction of thymidine kinase 1 after 5-fluorouracil as a mechanism for 3'-deoxy-3'-[18F]fluorothymidine flare. Biochem Pharmacol. 2010; 80:1528-1536.

9. Direcks WG, Berndsen SC, Proost N, Peters GJ, Balzarini J, Spreeuwenberg MD, Lammertsma AA and Molthoff CF. [18F]FDG and [18F]FLT uptake in human breast cancer cells in relation to the effects of chemotherapy: an *in vitro* study. Br J Cancer. 2008; 99:481-487.

10. Rasey JS, Grierson JR, Wiens LW, Kolb PD and Schwartz JL. Validation of FLT uptake as a measure of thymidine kinase-1 activity in A549 carcinoma cells. J Nucl Med. 2002; 43:1210-1217.

11. Ke PY, Kuo YY, Hu CM and Chang ZF. Control of dTTP pool size by anaphase promoting complex/cyclosome is essential for the maintenance of genetic stability. Genes Dev. 2005; 19:1920-1933.

12. Perumal M, Pillai RG, Barthel H, Leyton J, Latigo JR, Forster M, Mitchell F, Jackman AL and Aboagye EO. Redistribution of nucleoside transporters to the cell membrane provides a novel approach for imaging thymidylate synthase inhibition by positron emission tomography. Cancer Res. 2006; 66:8558-8564.

13. Plotnik DA, Emerick LE, Krohn KA, Unadkat JD and Schwartz JL. Different modes of transport for 3H-thymidine, 3H-FLT, and 3H-FMAU in proliferating and nonproliferating human tumor cells. J Nucl Med. 2010; 51:1464-1471.

14. Paproski RJ, Ng AM, Yao SY, Graham K, Young JD and Cass CE. The role of human nucleoside transporters in uptake of 3'-deoxy-3'-fluorothymidine. Mol Pharmacol. 2008; 74:1372-1380.

15. Paproski RJ, Wuest M, Jans HS, Graham K, Gati WP, McQuarrie S, McEwan A, Mercer J, Young JD and Cass CE. Biodistribution and uptake of 3'-deoxy-3'-fluorothymidine in ENT1-knockout mice and in an ENT1-knockdown tumor model. J Nucl Med. 2010; 51:1447-1455.

16. Pillai RG, Forster M, Perumal M, Mitchell F, Leyton J, Aibgirhio FI, Golovko O, Jackman AL and Aboagye EO. Imaging pharmacodynamics of the alpha-folate receptor-targeted thymidylate synthase inhibitor BGC 945. Cancer Res. 2008; 68:3827-3834.

17. Frings V, van der Veldt AA, Boellaard R, Herder GJ, Giovannetti E, Honeywell R, Peters GJ, Thunnissen E, Hoekstra OS and Smit EF. Pemetrexed Induced Thymidylate Synthase Inhibition in Non-Small Cell Lung Cancer Patients: A Pilot Study with 3'-Deoxy-3'-[(18)F]fluorothymidine Positron Emission Tomography. PLoS One. 2013; 8:e63705.

18. Hong YS, Kim HO, Kim KP, Lee JL, Kim HJ, Lee SJ, Oh SJ, Kim JS, Ryu JS, Moon DH and Kim TW. 3'-Deoxy-3'-18F-fluorothymidine PET for the early prediction of response to leucovorin, 5-fluorouracil, and oxaliplatin therapy in patients with metastatic colorectal cancer. J Nucl Med. 2013; 54:1209-1216.

19. Kenny LM, Contractor KB, Stebbing J, Al-Nahhas A, Palmieri C, Shousha S, Coombes RC and Aboagye EO. Altered tissue 3'-deoxy-3'-[18F]fluorothymidine pharmacokinetics in human breast cancer following capecitabine treatment detected by positron emission tomography. Clin Cancer Res. 2009; 15:6649-6657.

20. Wells P, Aboagye E, Gunn RN, Osman S, Boddy AV, Taylor GA, Rafi I, Hughes AN, Calvert AH, Price PM and Newell DR. 2-[11C]thymidine positron emission tomography as an indicator of thymidylate synthase inhibition in patients treated with AG337. J Natl Cancer Inst. 2003; 95:675-682.

21. Hong IK, Kim SY, Chung JH, Lee SJ, Oh SJ, Oh J, Ryu JS, Kim TW, Kim DY and Moon DH. 3'-Deoxy-3'-[18F]fluorothymidine positron emission tomography imaging of thymidine kinase 1 activity after 5-fluorouracil treatment in a mouse tumor model. Anticancer Res. 2014; 34:759-766.

22. Kaushal V, Mukunyadzi P, Siegel ER, Dennis RA, Johnson DE and Kohli M. Expression of tissue factor in prostate cancer correlates with malignant phenotype. Appl Immunohistochem Mol Morphol. 2008; 16:1-6.

23. Brockenbrough JS, Souquet T, Morihara JK, Stern JE, Hawes SE, Rasey JS, Leblond A, Wiens LW, Feng Q, Grierson J and Vesselle H. Tumor 3'-deoxy-3'-(18) F-fluorothymidine ((18)F-FLT) uptake by PET correlates with thymidine kinase 1 expression: static and kinetic analysis of (18)F-FLT PET studies in lung tumors. J Nucl Med. 2011; 52:1181-1188.

24. Plotnik DA, McLaughlin LJ, Chan J, Redmayne-Titley JN and Schwartz JL. The role of nucleoside/nucleotide transport and metabolism in the uptake and retention of 3'-fluoro-3'-deoxythymidine in human B-lymphoblast cells. Nucl Med Biol. 2011; 38:979-986.

25. Barthel H, Cleij MC, Collingridge DR, Hutchinson OC, Osman S, He Q, Luthra SK, Brady F, Price PM and Aboagye EO. 3'-deoxy-3'-[18F]fluorothymidine as a new marker for monitoring tumor response to antiproliferative therapy *in vivo* with positron emission tomography. Cancer Res. 2003; 63:3791-3798.

Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small cell lung cancer with FLT-PET imaging
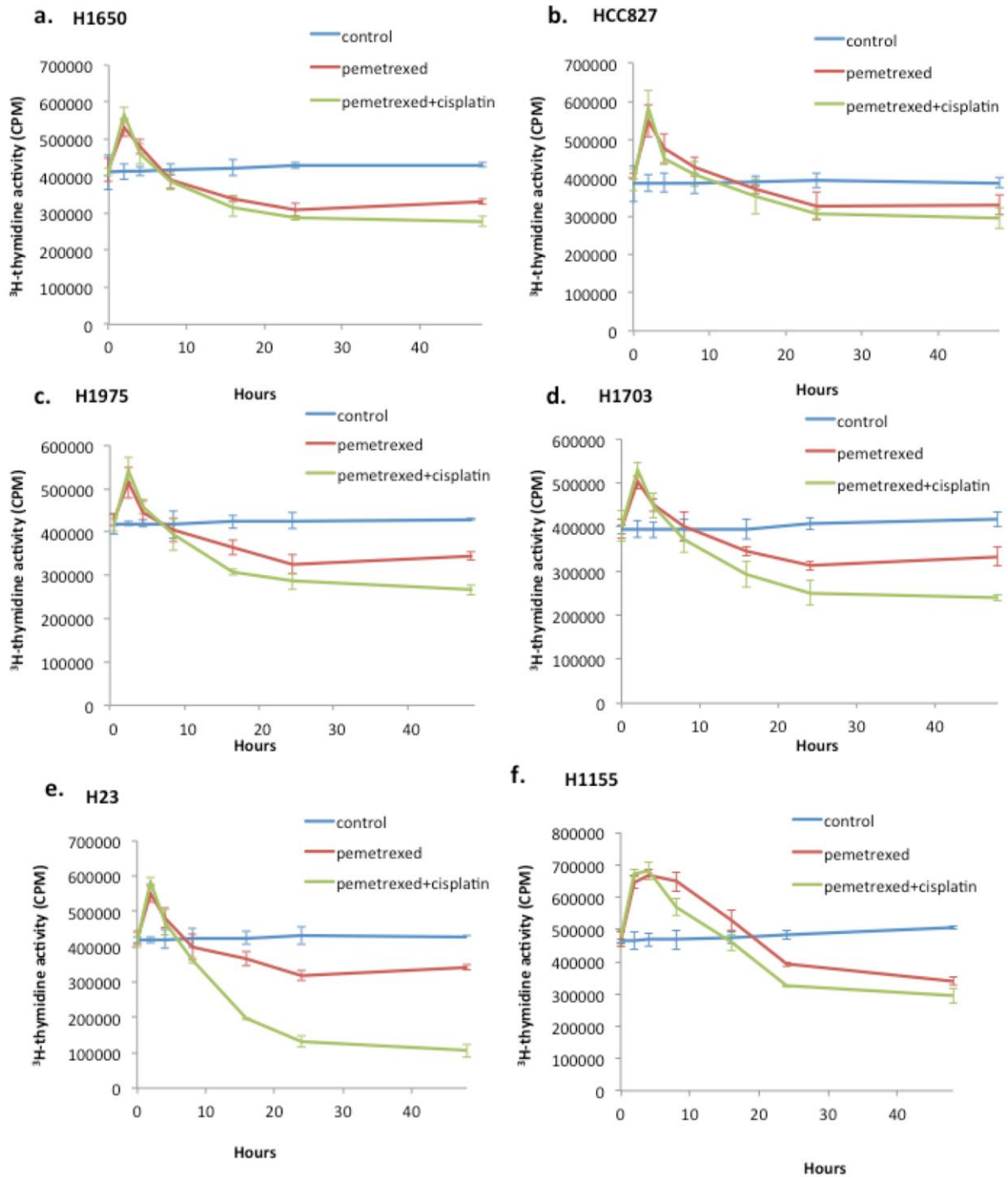
Supplementary Material

Supplemental Figure 1: Treatment of human NSCLC bearing xenografts with pemetrexed and cisplatin resulted in significant tumor growth inhibition. (a.) H460 bearing mouse xenografts were either treated as controls or given combination therapy with pemetrexed and cisplatin and tumors were measured over a 2 week course of therapy. (b., c.) Tumors were excised at necropsy demonstrating a significantly larger tumors in the (b.) control group relative to those (c.) treated with cisplatin and pemetrexed.

Cell lines	IC50 (μM)
H460	0.141
H1299	0.656
H1975	0.731
HCC827	0.175
H1650	0.182
H1703	0.618
H23	0.221
H1155	0.402

Supplemental Figure 2: IC50 calculations for pemetrexed sensitivity of NSCLC cell lines.

Supplemental Figure 3: Pemetrexed-induced TS inhibition results in a "flare" of the thymidine salvage pathway activity. 3H-thymidine assay was performed on PEM-sensitive NSCLC cell lines (a. -f.) in untreated control (culture medium only), pemetrexed (100nM) and combination therapy with pemetrexed (100nM) plus cisplatin (10mM). A "flare" in DNA salvage pathway activity was seen at 2 hrs of

exposure to pemetrexed in all 6 cell lines. Exposure to cisplatin showed no impact on the pemetrexed induced "flare".

Early detection of thymidylate synthase resistance in non-small cell lung cancer with FLT-PET imaging

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ABSTRACT

Introduction: Inhibition of thymidylate synthase (TS) results in a transient compensatory "flare" in thymidine salvage pathway activity measureable with ^{18}F-thymidine (FLT)- positron emission tomography (PET) at 2hrs. of therapy which may predict non-small cell lung cancer (NSCLC) sensitivity to TS inhibition.

Materials and Methods: Resistance to TS inhibition bypemetrexed was induced in NSCLC cell lines H460 and H1299 through TS overexpression. TS overexpression was confirmed with RT-PCR and Western blotting and pemetrexed resistance confirmed with IC_{50} assays. The presence of a pemetrexed-induced thymidine salvage pathway "flare" was then measured using ^{3}H-thymidine in both pemetrexed sensitive (H460 and H1299) and resistant (H460R, H1299R, CALU-6, H522, H650, H661, H820, H1838) lines *in vitro*, and validated with FLT-PET *in vivo* using H460 and H460R xenografts.

Results: Overexpression of TS induced pemetrexed resistance with IC_{50} for H460, H1299, H460R and H1299R measured as 0.141 μM, 0.656 μM, 22.842 μM, 213.120 μM, respectively. Thymidine salvage pathway ^{3}H-thymidine "flare" was observed following pemetrexed in H460 and H1299 but not H460R, H1299R, CALU-6, H522, H650, H661, H820 or H1838 *in vitro*. Similarly, a FLT "flare" was observed *in vivo* following pemetrexed therapy in H460 but not H460R tumor-bearing xenografts.

Conclusions: Imaging of TS inhibition is predictive of NSCLC sensitivity to pemetrexed.

INTRODUCTION

Inhibitors of thymidylate synthase (TS), a key enzyme in the *de novo* thymidine synthesis pathway, play a role in the treatment of a number of malignancies including non-small cell lung cancer (NSCLC). Examples of commonly used TS inhibitors in cancer therapy include 5-fluorouracil (5-FU), pemetrexed and capecitabine. Successful inhibition of TS results in a transient compensatory "flare" in activity of the thymidine salvage pathway [1–4], which also sources thymidine to the dividing cell. As a result, this drug-induced compensatory

"flare" in thymidine salvage pathway activity is an indicator of successful TS inhibition. This drug-induced change in tumor metabolism can be made visible through ^{18}F-thymidine (FLT)-positron emission tomography (PET) [2, 5–7], an analog of thymidine. FLT, first described by Shields in 1998 [8], is an investigational imaging biomarker of the thymidine salvage pathway currently in use for human clinical trials primarily as a validated surrogate marker of tumor proliferation [9–12]. Here we consider FLT-PET imaging as a means of detecting successful TS targeting by pemetrexed, a TS inhibitor currently in use for NSCLC therapy.

In our recent publication [7], we characterized the kinetics of the pemetrexed-induced thymidine salvage pathway "flare" in a xenograft mouse model of human NSCLC and demonstrated that the peak of the pemetrexed-induced thymidine salvage pathway "flare" consistently occurs at 2 hours of exposure to therapy both *in vitro* and *in vivo*. Beyond that 2 hour time point, the pemetrexed-induced "flare" signal decays, dissipating entirely by 24 hours of therapy at which time the anti-proliferative effects of successful therapy have begun to dominate thymidine salvage pathway activity. We also have determined that the pemetrexed-induced "flare" effect is mediated in part by rapid changes in the activity of thymidine kinase 1 (TK1), the key rate-limiting step in the thymidine salvage pathway enzyme and on the mobilization of equilibrative nucleoside transporter 1 (ENT 1) to the cell surface. ENT1 has been shown to be important for facilitation of thymidine, and thymidine analogues, entry into proliferating cells [13–16]. These findings are consistent with published literature also demonstrating that increases in TK1 activity and/or ENT1 mobilization play a significant role in the TS-inhibition mediated thymidine salvage pathway "flare" in activity [2, 3, 17–19].

The question remains whether this TS inhibitor-induced thymidine salvage pathway "flare" in tumor metabolism can serve as a reliable indicator of therapy success with eventual tumor regression. While there is literature describing the phenomenon the TS-inhibition induced "flare" in the salvage pathway activity, there is very little available data examining whether this strategy can be predictive of drug therapy success in cancer with several published papers yielding mixed results [20, 21]. Therefore careful pre-clinical study is warranted to determine whether the pemetrexed-induced "flare" can be predictive of therapy success in NSCLC. Previously, our laboratory has examined the thymidine salvage pathway "flare" imaging strategy in the setting of pemetrexed sensitivity [7]; we now characterize the "flare" in the setting of pemetrexed resistance. TS overexpression is a common and well-described mechanism of cancer resistance to TS inhibitors including NSCLC resistance to pemetrexed [22–25]. In this study we employ TS overexpression to induce resistance to TS inhibition in order to study the pemetrexed-induced "flare" in thymidine salvage pathway activity in the setting of NSCLC drug resistance.

RESULTS

Overexpression of TS is associated with resistance to pemetrexed in NSCLC lines *in vitro*

Overexpression of TS in H460 and H1299 cell lines was confirmed *in vitro* using RT-PCR, Western blotting and immunofluorescence (Figure 1 and Supplementary Figure 1). IC_{50} measurements confirmed pemetrexed sensitivity in the wild-type H460 and H1299 cell lines and the development of pemetrexed resistance in the TS overexpressing H460R and H1299R cell lines with IC_{50} measurements of 0.141 μM, 22.8 μM, 0.656 μM, 213 μM for H460, H460R, H1299 and H1299R respectively (Supplementary Figure 2).

In addition, the TS protein expression was also noted to be elevated in six inherently pemetrexed resistant NSCLC cell lines CALU6, H522, H650, H661, H820, H1838 relative to pemetrexed-sensitive cell lines H460 and H1299 (Supplementary Figure 1) with IC50 measurements of (Supplementary Figure 2). The IC50 for CALU6, H522, H650, H661, H820, H1838 were 14.2, 59.6, 238, 85.0, 48.0, 121 μM respectively.

Overexpression of TS induces resistance to pemetrexed in NSCLC lines H460 and H1299 *in vivo*

Xenografts bearing H460 (16 mice) or H460R (16 mice) were treated as either vehicle only controls (8 mice per cell line) or given combination therapy with pemetrexed and cisplatin (8 mice per cell line) for 2 weeks. Routine external caliper tumor measurements were compared between treated and control groups for each cell line. A significant tumor growth inhibition was observed in the pemetrexed-sensitive H460 chemotherapy treated tumors (74.7±15.1%; p=0.0002) relative to untreated vehicle only controls. There was a slight tumor growth inhibition in the treated pemetrexed-resistant H460R tumors when compared to vehicle only treated controls (47.8±14.4%, p=0.0001) (Supplementary Figure 3).

Induction of pemetrexed resistance through TS overexpression results in loss of thymidine salvage pathway "flare" *in vitro*

Thymidine salvage pathway activity was assayed with ^{3}H-thymidine at baseline and following treatment with either pemetrexed, cisplatin or combination of cisplatin and pemetrexed *in vitro*. A "flare" in thymidine salvage pathway activity measured in pemetrexed-sensitive NSCLC lines H460 (43.4±5.11%, p=0.0005) and H1299 (39.5±6.51%, p<0.0001) but not in TS overexpressed pemetrexed-resistant NSCLC cell lines H460R (-2.44±0.95%, p=0.421) and H1299R (-0.60±4.40%, p=0.870) *in vitro* using ^{3}H-thymidine assays (Figure 2). In addition, none of the six lines inherently NSCLC resistant NSCLC cells had a pemetrexed-induced thymidine salvage pathway "flare" at 2 hours (CALU6: -1.87±7.20%, p=0.742; H522: 7.63±6.00%, p=0.115; H650: -1.73±3.99%, p=0.454; H661: 3.82±5.13%, p=0.201; H820: -0.03±3.37%, p=0.958; H1838: 3.21±5.20%, p=0.250) (Figure 3). One cell line, CALU6,

had a delayed "flare" at 24 hours following start of exposure to pemetrexed therapy (66.9±8.08%, p=0.0005).

Induction of pemetrexed resistance through TS overexpression results in loss of thymidine salvage "flare" *in vivo*

A total of 4 groups of xenografted mice were imaged with FLT-PET at baseline and at 2 hours of therapy and changes in tumor avidity for FLT were measured. These groups were as follows: H460 (8 mice; vehicle only), H460R (8 mice; vehicle only), H460 (8 mice; cisplatin/pemetrexed), H460R (8 mice; cisplatin/pemetrexed). A FLT "flare" in tumor avidity was observed at 2 hours of therapy for pemetrexed-sensitive H460 xenografts (58.6 ±16.1% increase over baseline; p=0.043) but not pemetrexed-resistant H460R xenografts (10.8 ±7.28% increase over baseline; p=0.653)

(Figure 4). No FLT "flare" was observed in the vehicle-only controls for H460 (1.44±6.35% increase over baseline; p=0.9582) or H460R (2.34±11.86% increase over baseline ; p=0.9527).

DISCUSSION

Tumor resistance to pemetrexed, a TS inhibitor used in the treatment of NSCLC, is a common problem in oncologic management. Successful TS inhibition results in a transient "flare" in tumor cellular thymidine salvage pathway activity that is measurable *in vitro* and *in vivo* with FLT-PET imaging. In a previous publication, we described the optimal timing of imaging the TS-inhibition mediated "flare" in the thymidine salvage pathway and detailed mechanisms underlying this effect including increasing of TK1 activity and mobilization of ENT1 to
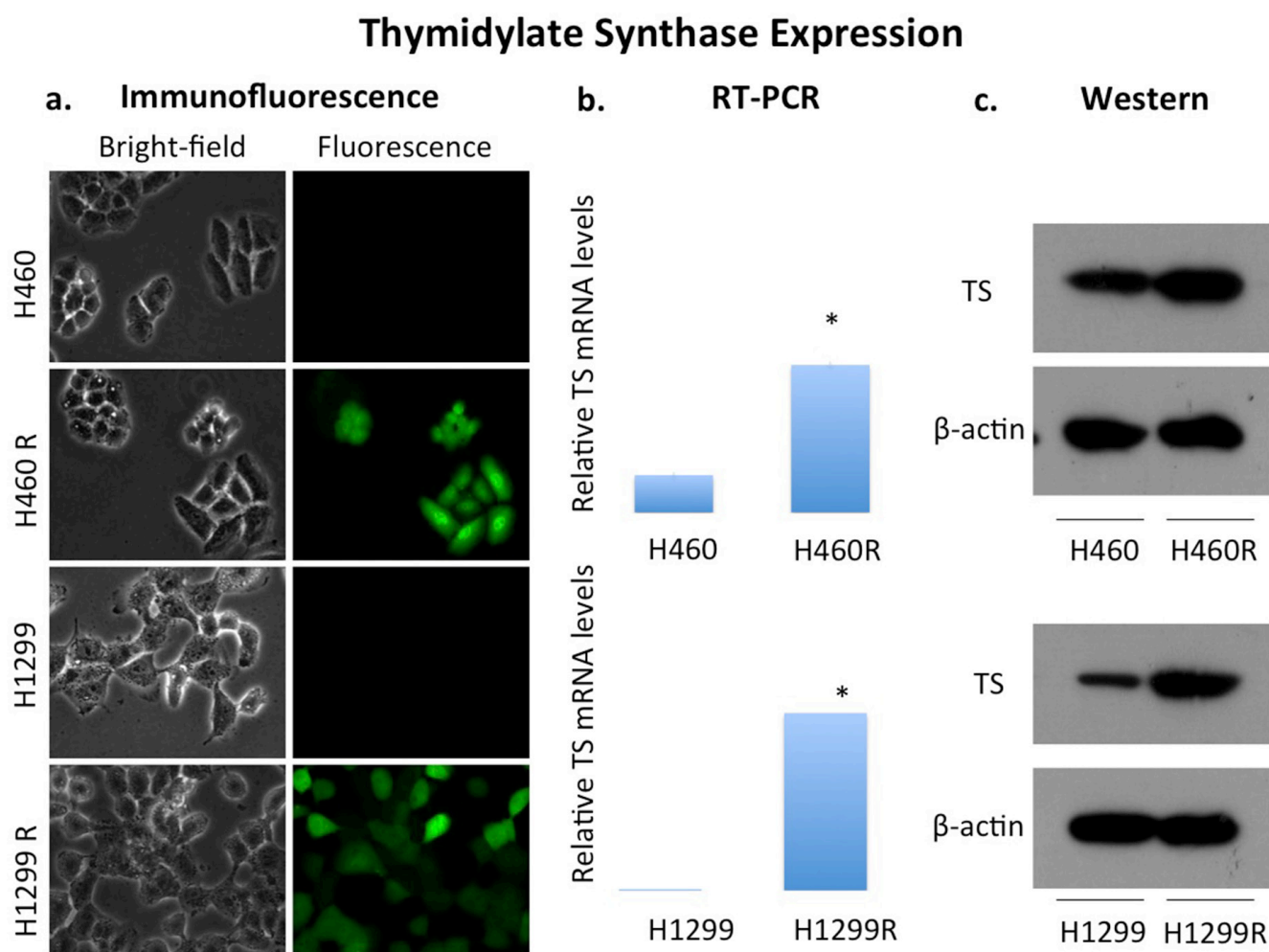

Figure 1: Overexpression of thymidylate synthase in NSCLC cell lines H460 and H1299. Pemetrexed sensitive human NSCLC cell lines H460 and H1299 expressed thymidylate synthase (TS) at low levels at baseline. Overexpression of TS in both cell lines, termed H460R and H1299R, was confirmed with **(a)** detection of GFP in transfected cells by immunofluorescence (TS) **(b)** quantification of TS mRNA by RT-PCR and **(c)** protein expression.

the cell surface [7]. In this study we further examine this imaging technique to determine if the presence of the TS-inhibition induced "flare" in the thymidine salvage pathway is predictive of NSCLC tumor sensitivity to pemetrexed.

Since overexpression of TS is a common mechanism of tumor resistance to TS-inhibition, we chose to study the impact of pemetrexed resistance on the thymidine salvage pathway "flare" by overexpressing TS in two previously sensitive NSCLC cell lines, H460 and H1299. All examined pemetrexed-resistant NSCLC cell lines, those created though overexpression of TS and inherently resistant cell lines, demonstrated elevated levels of TS protein expression relative to the pemetrexed sensitive NSCLC cell lines, H460 and H1299. This is in keeping with published literature demonstrating that TS overexpression is a common mechanism for resistance to pemetrexed therapy in NSCLC [22].

Following overexpression of TS, H460R and H1299R cell lines demonstrated the expected resistance to treatment with pemetrexed as measured by IC_{50} assays *in vitro* and relative tumor growth inhibition *in vivo*. The pemetrexed-resistant H460R xenografts still demonstrated some tumor growth inhibition in response to chemotherapy as a result of sensitivity to cisplatin. Combination therapy with cisplatin and pemetrexed therapy was administered in order to model the regimen typically administered to NSCLC patients. Even so, the absence of thymidine salvage pathway FLT-PET "flare" *in vivo* was able to correctly identify tumors exhibiting a suboptimal tumor response to therapy due to pemetrexed resistance.

Two pemetrexed-sensitive human NSCLC cell lines, H460 and H1299, both exhibit a TS-inhibition mediated thymidine salvage pathway flare at 2 hours of exposure to pemetrexed. With induction of pemetrexed-resistance through TS overexpression, both H460R and H1299R cell lines reveal a loss of the TS-inhibition mediated thymidine
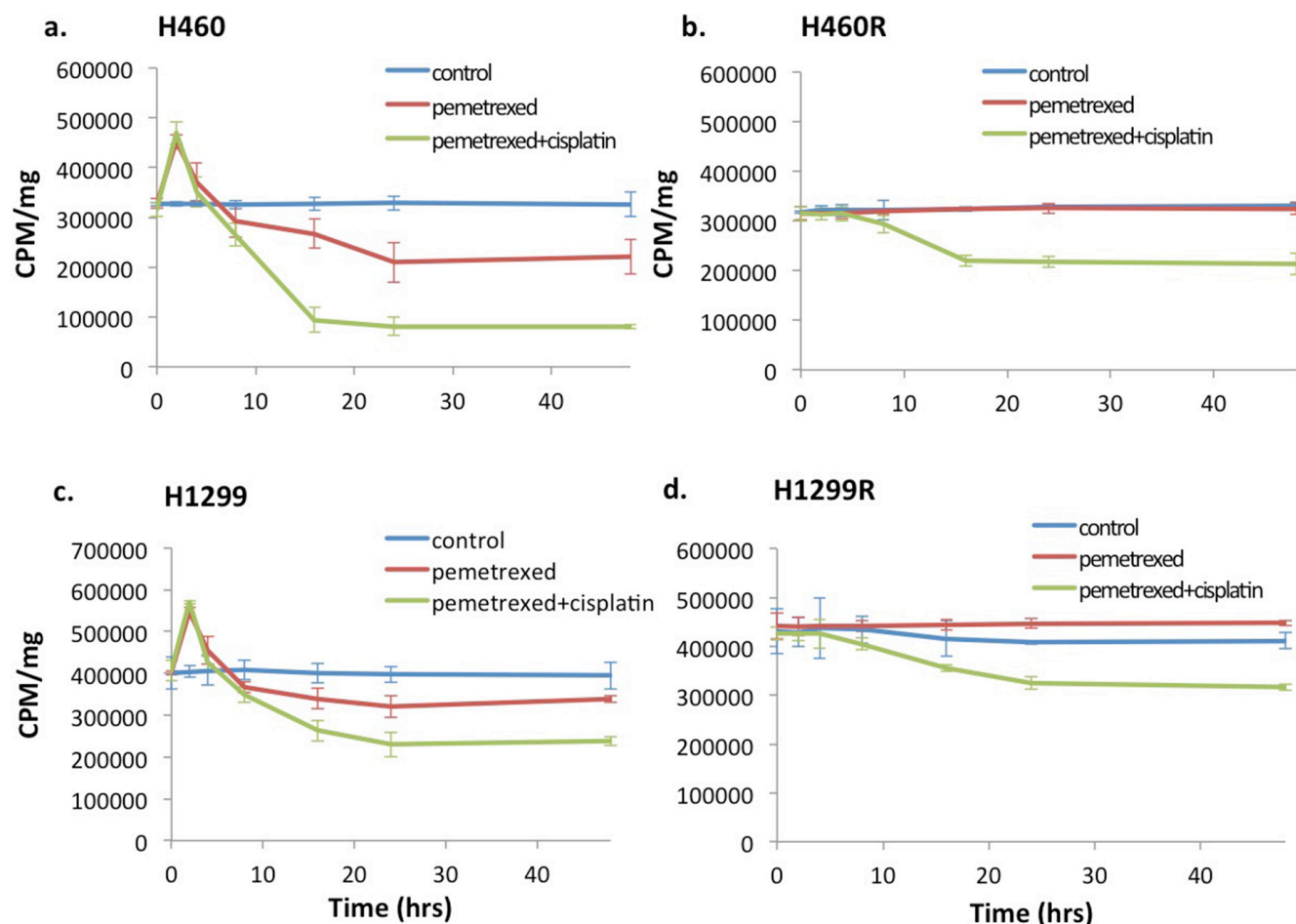

Figure 2: Pemetrexed-resistance conferred by TS overexpression results in loss of the pemetrexed-induced DNA salvage pathway "flare" in NSCLC *in vitro*. Pemetrexed-sensitive wild type NSCLC cell lines H460 **(a)** and H1299 **(c)** demonstrated a "flare" in thymidine salvage pathway on 3H-thymidine assay at 2 hrs. of exposure to pemetrexed. The overexpression of TS eliminated the pemetrexed-induced "flare" in thymidine salvage pathway activity in both cell lines, H460R **(b)** and H1299R **(d)**. The exposure to cisplatin did not impact the presence of the pemetrexed-induced thymidine salvage pathway "flare".
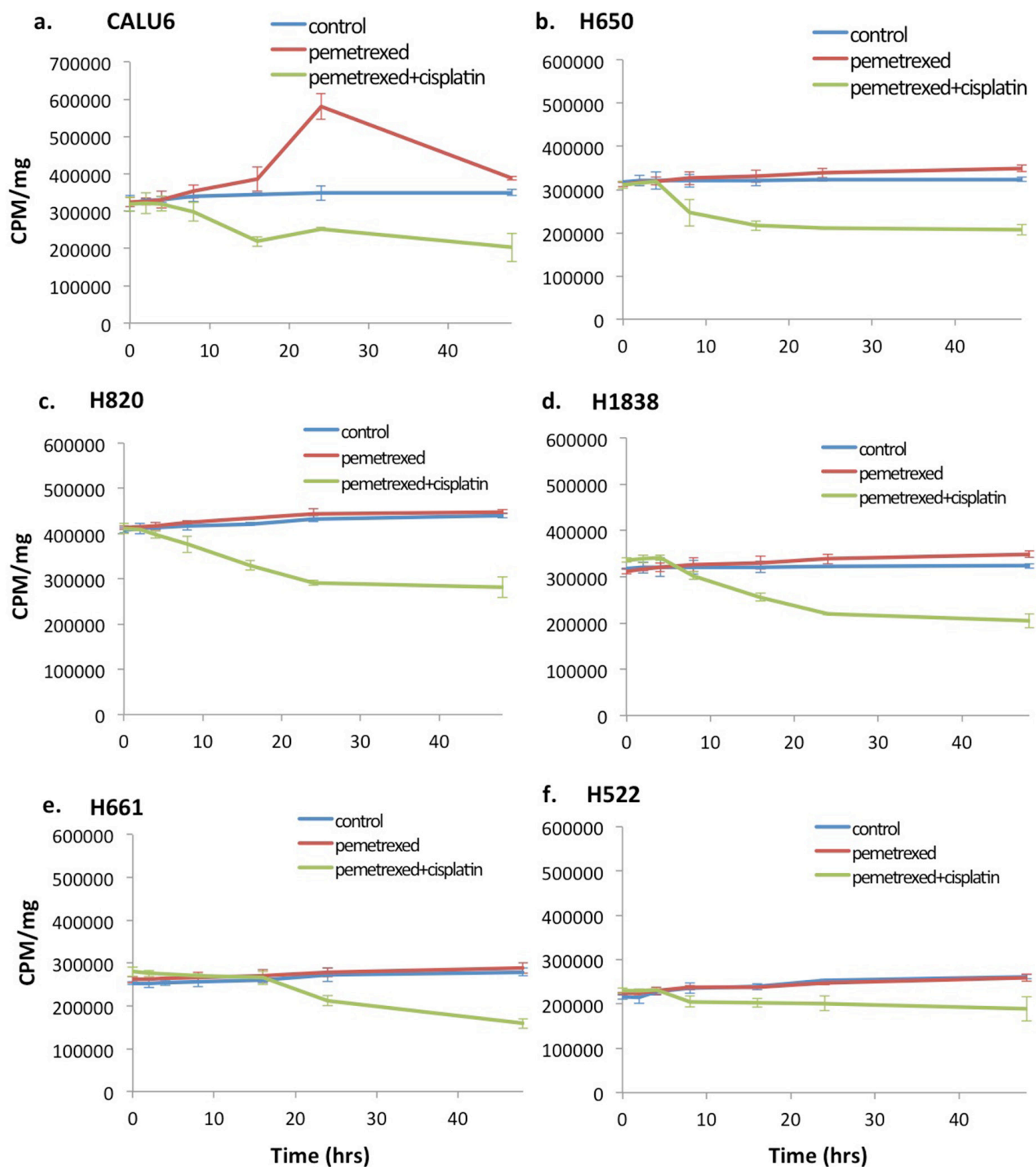
Figure 3: Wild-type resistance to pemetrexed in NSCLC cell lines results in loss of the thymidine salvage pathway induced "flare" at the 2hr time point. 3H-thymidine assay was performed on inherently pemetrexed-resistant NSCLC cell lines **(a-f)** following incubation without chemotherapy (culture medium only), pemetrexed (100 nM) or combination therapy with pemetrexed (100 nM) plus cisplatin (10mM). No "flare" in thymidine salvage pathway activity was seen at 2 hrs…. of exposure to pemetrexed in any of the 6 pemetrexed-resistant cell lines. One cell line, CALU6 **(a)**, demonstrated a delayed increase in thymidine pathway activity occurring at approximately 24 hrs. of therapy. Exposure to cisplatin showed no impact on the pemetrexed induced "flare".

salvage pathway "flare" at 2 hours *in vitro* and *in vivo*. This loss of the thymidine salvage pathway "flare" was also observed in a panel of inherently pemetrexed-resistant wild-type NSCLC cell lines. Interesting, one of these resistant cell lines, CALU-6, exhibited a delayed increase in thymidine salvage pathway at 24 hours of exposure to therapy. We hypothesize that this delayed increased in thymidine salvage pathway activity at 24 hours may be on the basis of TS-inhibitor-induced increased expression of TK1 as previously reported [2]. It should be noted that in this study we did not folate restrict the murine diet or administer intravenous thymidine phosphorylase prior to imaging, which is sometimes done to prevent saturation of TS or FLT by high murine endogenous levels of folate or thymidine respectively. As a result, our data may underestimate the magnitude of the pemetrexed induced FLT "flare" in NSCLC.

In summary, here we demonstrate evidence that the presence of a thymidine salvage pathway "flare" at 2 hours may be predictive of tumor response to pemetrexed therapy in a preclinical model of human NSCLC. In this study we demonstrated the absence of a TS-inhibition induced "flare" in thymidine salvage pathway activity in all of the 8 pemetrexed-resistant NSCLC cell lines. The data presented here is complementary to our previous report demonstrating the presence of a TS-inhibition mediated thymidine salvage pathway "flare" at 2 hours in all of the 8 pemetrexed-sensitive human NSCLC cell lines examined. Although there is a published clinical pilot study of the FLT "flare" in NSCLC which did not demonstrate a predictive value of this technique [20], it is plausible that other variables such as concurrent treatment with dexamethasone, known to decrease the expression of thymidylate synthase and dihydrofolate reductase and impact tumor responsiveness to pemetrexed [26], could impact the translation of this technique into the clinic. Careful study is needed to determine if the TS-Inhibition mediated FLT "flare" could be of value in the clinical setting. If successful, this technique has the potential of
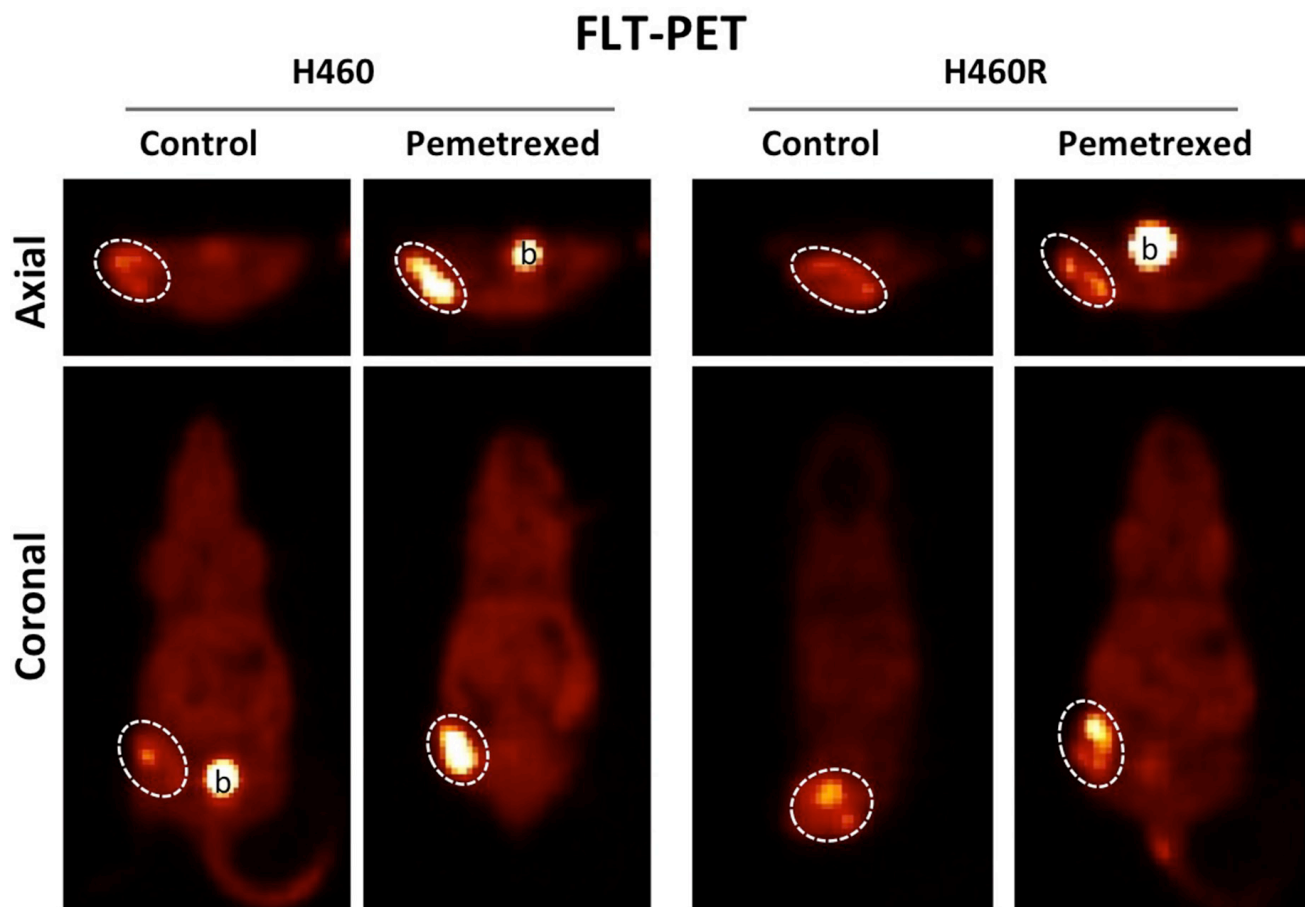

Figure 4: Pemetrexed-resistance conferred by TS overexpression results in loss of the pemetrexed-induced thymidine salvage pathway "flare" in NSCLC *in vivo*. FLT-PET imaging was performed on pemetrexed-sensitive H460 and pemetrexed-resistant H460R xenografts the day before and 2 hours after exposure to combination therapy with pemetrexed and cisplatin. A pemetrexed-induced "flare" in thymidine salvage pathway activity is observed in the pemetrexed sensitive H460 xenografts but not the resistant H460R xenografts.

determining NSCLC sensitivity to pemetrexed therapy as early as the day of therapy start.

MATERIALS AND METHODS

Chemotherapeutics and imaging radiopharmaceuticals

For *in vitro* studies, pemetrexed (Santa Cruz Biotechnology, Dallas, Texas) and cisplatin (Sigma-Aldrich Corp., St. Louis, MO) were provided in solid form, dissolved in water and stored at -20°C as a 0.2 mM and 1 mM stock, respectively. For *in vivo* use, both human and murine, pemetrexed (ALIMTA; Eli Lilly and Company, Indianapolis, IN) and cisplatin (Teva Pharmaceuticals, PetachTikva Israel) were provided freshly prepared as a 1 mg/ml sterile saline solution by the Abramson Cancer Center Pharmacy. For *in vivo* murine studies, chemotherapeutics were stored at 4°C. [18F]FLT was produced on site in the University of Pennsylvania PET Center Cyclotron facility. [18F]FLT average specific activity was 5.32 +/- 2.14 Ci/umol, and radiochemical purity >99%.

Cell lines and culture

All human non-small cell lung cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in RPMI medium containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator in 5% CO_2 at 37°C. Passage of cell lines was performed at 1:3 dilution after detachment using sterile 0.05% trypsin-EDTA solution.

IC_{50} calculations

Cultured cell lines were harvested and seeded into a 24-well plate (2×10^4 cells per well) in RPMI1640 culture medium and incubated for 24 hours at 37°C in a 5% CO_2 incubator. The culture medium was then replaced with 100 uL of fresh medium containing varying concentrations of pemetrexed (0, 0.01, 0.1, 1, 10, 100 μM) and incubated for 72 hours at 37°C in a 5% CO_2 incubator. The IC_{50} assay was performed then performed using the MTT Cell Growth Assay Kit (Sigma-Aldrich, St. Louis, MO). Absorbance of the converted dye was measured using a Beckman DU-600 Spectrophotometer (Beckman Coulter Life Sciences, Indianapolis, IN) and data analyzed using the statistical software SPSS 19.0 (IBM, Chicago, USA).

Mouse tumor xenograft modeling

Prior to *in vivo* animal modeling, approval was obtained by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

Human tumor-bearing murine xenografts were then created using two month-old female *nu/nu* mice (Crl: NUFoxnlnu, Charles River Laboratory, Wilmington, MA) and fed a conventional murine diet. Each mouse was injected subcutaneously in the flank with a suspension of H460 cells (5×10^6) in sterile, endotoxin-free 50% Matrigel Matrix (Corning Inc., Corning, NY). When tumors reached a mean volume of approximately 200 mm^3 (volume= π/6 × length × width × height), animals were randomized into treatment groups.

³H-thymidine assays

[³H]-thymidine (Perkin Elmer NET355001MC, PerkinElmer, Waltham, MA) was utilized for *in vitro* assessment of therapy-induced changes in thymidine salvage pathway activity in cultured human NSCLC cells. [³H]-thymidine specific activity was >10Ci(370GBq)/mmol and radiochemical purity >97%. H460 and H1299 cells were seeded (1×10^6/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 hours in 5% CO_2 at 37°C. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM) and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48 hours. Drug-containing medium was then removed, and the cells were then washed and pulsed with 5 μCi [³H]-thymidine/well for 1 hour. The cells were then washed and scraped into plastic vials. Scintillant (10 mL; Research Products International Corp., Mount Prospect, IL) was added to each vial and the radioactivity was counted on a scintillation counter (Beckman Coulter LS6500, Beckman Coulter Life Sciences, Indianapolis, IN).

Gene overexpression

A full-length cDNA fragment encoding TS was obtained from pDONR223-DTYMK plasmid (Addgene) by polymerase chain reaction with the primers TS-F (5'-ATCCCGGGCCTTGAGCGGCCCGGCGCGGG-3') and TS-R (5'-CTCCGGAACGAATTCTCACTTCCA TAGCTC-3'), and subcloned into the Xbal and EcoR I sites of lentiviral vector pUltra (Addgene). The product was verified by sequencing. Lentivirus was packaged by transfecting into HEK 293T cells with the packaging plasmids pMD2-VSV.G and pCMV-dR8.74. Virus-containing medium was harvested 48 hours and 72 hours after transfection and filtered with 0.45 μm Millex HV filters (EMD Millipore). Lenti-X Concentrator (Clontech) and virus-containing medium (v/v: 1:3) were mixed and incubated at 4°C for 1 hour. The viral particles were concentrated by centrifugation at 1,500 x g for 45 minutes at 4°C. The resulting pellet was then suspended in fresh

RPMI 1640 medium and used to infect H460 and H1299 cells.

Immunoblotting

Therapy-induced changes in the protein expression of key components of the thymidine salvage pathway were assessed using Western blot analysis. H460 and H1299 cells were seeded (1×10^6 cells/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 hour in 5% CO_2 at 37°C. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM) and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48 hours. Whole cell lysates were then generated using a 1% Nonidet P-40 lysis buffer (Sigma-Aldrich Corp., St. Louis, MO). The suspension was homogenized by passages through a 20-gauge syringe needle and nuclear material removed through centrifugation at 14000 rpm for 15 min at 4°C. Cell lysates were then loaded onto a precast Nupage Bis-Tris Gels (invitrogen, Life Technologies, Corp., Grand Island, NY) for electrophoresis then transferred onto Hybond-P PVDF membranes (Sigma-Aldrich Corp., St. Louis, MO) for Western blot analysis. After blocking membranes with 5% non-fat milk in PBS with 0.1% Tween-20 buffer, PVDF membranes were probed using primary antibodies directed against human TK1 (Cell Signaling Technology, Danvers, MA; 1:5000), human TS (Cell Signaling Technology; 1:4000), or human β-actin (Sigma-Aldrich Corp., St. Louis, MO; 1:10000). Membrane were then washed and incubated (1:3000) with species-specific secondary antibodies, either anti-rabbit or anti-mouse, conjugated to horseradish peroxidase (GE Healthcare Life Science; 1:3000) for 1 hour, the proteins were detected using the Immobilon ECL system (EMD Millipore, Billerica, MA) and quantified using Image J software available through the National Institutes of Health (https://imagej.nih.gov/ij/index.html).

PET imaging

Baseline FLT-PET scans were performed on the day prior to therapy. Mice were then treated with either the vehicle control (i.p. sterile PBS) or combination therapy with pemetrexed (i.p. 100 mg/kg) and cisplatin (10 mg/kg). A post-therapy FLT-PET was then performed at varying time points following administration of therapy.

For the assessment tumor response to therapy, tumor-bearing animals were treated for a period of two weeks with 100 mg/kg pemetrexed (i.p. daily; days 1-5 and 8-12) and 10 mg/kg cisplatin (i.p. once weekly). During this treatment period, tumor volumes were estimated by external caliper measurements. After therapy/imaging completion, mice were euthanized with carbon dioxide inhalation.

After anesthesia with inhaled isofluorane in O_2 (3% induction, 1.5% maintenance), mice were injected intravenously with 300-350 μCi of [18F]FLT then allowed to r``ecover from the anesthesia during the 60-min uptake time allowed for radiotracer accumulation. At 60 min post-injection, mice were anesthetized and imaged for a 15-min static acquisition on the small animal PET scanner (A-PET, built in collaboration with Philips Medical Systems) located at the University of Pennsylvania Small Animal Imaging Facility.

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

FUNDING

REFERENCES

1. Kenny LM, Contractor KB, Stebbing J, Al-Nahhas A, Palmieri C, Shousha S, Coombes RC, Aboagye EO. Altered tissue 3'-deoxy-3'-[18F]fluorothymidine pharmacokinetics in human breast cancer following capecitabine treatment detected by positron emission tomography. Clin Cancer Res. 2009; 15:6649-6657.

2. Lee SJ, Kim SY, Chung JH, Oh SJ, Ryu JS, Hong YS, Kim TW, Moon DH. Induction of thymidine kinase 1 after 5-fluorouracil as a mechanism for 3'-deoxy-3'-[18F]fluorothymidine flare. Biochem Pharmacol. 2010; 80:1528-1536.

3. Pressacco J, Mitrovski B, Erlichman C, Hedley DW. Effects of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. Cancer Res. 1995; 55:1505-1508.

4. Wells P, Aboagye E, Gunn RN, Osman S, Boddy AV, Taylor GA, Rafi I, Hughes AN, Calvert AH, Price PM, Newell DR. 2-[11C]thymidine positron emission tomography as an indicator of thymidylate synthase inhibition in patients treated with AG337. J Natl Cancer Inst. 2003; 95:675-682.

5. McHugh CI, Lawhorn-Crews JM, Modi D, Douglas KA, Jones SK, Mangner TJ, Collins JM, Shields AF. Effects of capecitabine treatment on the uptake of thymidine analogs using exploratory PET imaging agents: 18F-FAU, 18F-FMAU, and 18F-FLT. Cancer Imaging. 2016; 16:34.

6. Heskamp S, Heijmen L, Gerrits D, Molkenboer-Kuenen JD, Ter Voert EG, Heinzmann K, Honess DJ, Smith DM, Griffiths JR, Doblas S, Sinkus R, Laverman P, Oyen WJ, Heerschap A, Boerman OC. Response Monitoring with [18F]FLT PET and Diffusion-Weighted MRI After
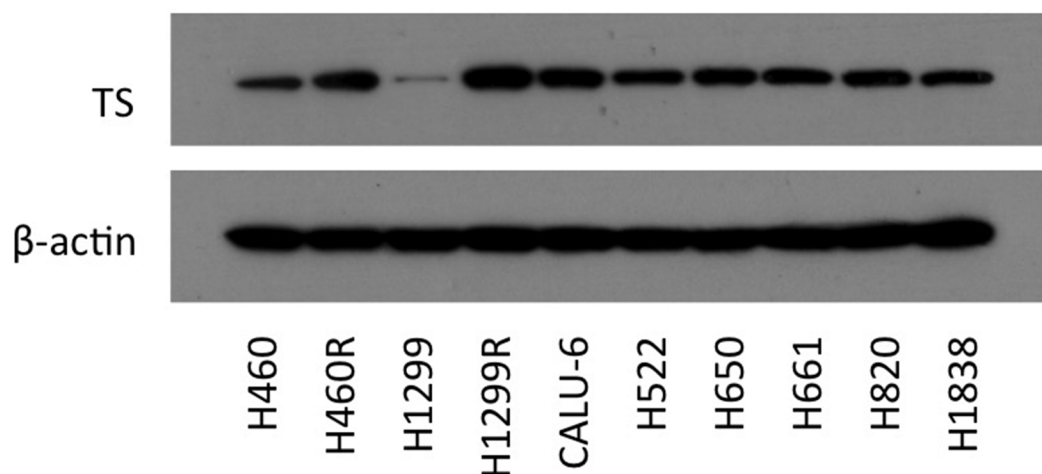
Cytotoxic 5-FU Treatment in an Experimental Rat Model for Colorectal Liver Metastases. Mol Imaging Biol. 2016.

7. Chen X, Yang Y, Berger I, Khalid U, Patel A, Cai J, Farwell MD, Langer C, Aggarwal C, Albelda SM, Katz SI. Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small cell lung cancer with FLT-PET imaging. Oncotarget. 2016.

8. Shields AF, Grierson JR, Dohmen BM, Machulla HJ, Stayanoff JC, Lawhorn-Crews JM, Obradovich JE, Muzik O, Mangner TJ. Imaging proliferation *in vivo* with [F-18] FLT and positron emission tomography. Nat Med. 1998; 4:1334-1336.

9. Barthel H, Cleij MC, Collingridge DR, Hutchinson OC, Osman S, He Q, Luthra SK, Brady F, Price PM, Aboagye EO. 3'-deoxy-3'-[18F]fluorothymidine as a new marker for monitoring tumor response to antiproliferative therapy *in vivo* with positron emission tomography. Cancer Res. 2003; 63:3791-3798.

10. Muzi M, Vesselle H, Grierson JR, Mankoff DA, Schmidt RA, Peterson L, Wells JM, Krohn KA. Kinetic analysis of 3'-deoxy-3'-fluorothymidine PET studies: validation studies in patients with lung cancer. J Nucl Med. 2005; 46:274-282.

11. Rasey JS, Grierson JR, Wiens LW, Kolb PD, Schwartz JL. Validation of FLT uptake as a measure of thymidine kinase-1 activity in A549 carcinoma cells. J Nucl Med. 2002; 43:1210-1217.

12. Vesselle H, Grierson J, Muzi M, Pugsley JM, Schmidt RA, Rabinowitz P, Peterson LM, Vallieres E, Wood DE. *In vivo* validation of 3'deoxy-3'-[(18)F]fluorothymidine ([(18)F]FLT) as a proliferation imaging tracer in humans: correlation of [(18)F]FLT uptake by positron emission tomography with Ki-67 immunohistochemistry and flow cytometry in human lung tumors. Clin Cancer Res. 2002; 8:3315-3323.

13. Paproski RJ, Wuest M, Jans HS, Graham K, Gati WP, McQuarrie S, McEwan A, Mercer J, Young JD, Cass CE. Biodistribution and uptake of 3'-deoxy-3'-fluorothymidine in ENT1-knockout mice and in an ENT1-knockdown tumor model. J Nucl Med. 2010; 51:1447-1455.

14. Paproski RJ, Ng AM, Yao SY, Graham K, Young JD, Cass CE. The role of human nucleoside transporters in uptake of 3'-deoxy-3'-fluorothymidine. Mol Pharmacol. 2008; 74:1372-1380.

15. Plotnik DA, Emerick LE, Krohn KA, Unadkat JD, Schwartz JL. Different modes of transport for 3H-thymidine, 3H-FLT, and 3H-FMAU in proliferating and nonproliferating human tumor cells. J Nucl Med. 2010; 51:1464-1471.

16. Plotnik DA, McLaughlin LJ, Chan J, Redmayne-Titley JN, Schwartz JL. The role of nucleoside/nucleotide transport and metabolism in the uptake and retention of 3'-fluoro-3'-deoxythymidine in human B-lymphoblast cells. Nucl Med Biol. 2011; 38:979-986.

17. Perumal M, Pillai RG, Barthel H, Leyton J, Latigo JR, Forster M, Mitchell F, Jackman AL, Aboagye EO. Redistribution of nucleoside transporters to the cell membrane provides a novel approach for imaging thymidylate synthase inhibition by positron emission tomography. Cancer Res. 2006; 66:8558-8564.

18. Pillai RG, Forster M, Perumal M, Mitchell F, Leyton J, Aibgirhio FI, Golovko O, Jackman AL, Aboagye EO. Imaging pharmacodynamics of the alpha-folate receptor-targeted thymidylate synthase inhibitor BGC 945. Cancer Res. 2008; 68:3827-3834.

19. Plotnik DA, McLaughlin LJ, Krohn KA, Schwartz JL. The effects of 5-fluorouracil treatment on 3'-fluoro-3'-deoxythymidine (FLT) transport and metabolism in proliferating and non-proliferating cultures of human tumor cells. Nucl Med Biol. 2012; 39:970-976.

20. Frings V, van der Veldt AA, Boellaard R, Herder GJ, Giovannetti E, Honeywell R, Peters GJ, Thunnissen E, Hoekstra OS, Smit EF. Pemetrexed induced thymidylate synthase inhibition in non-small cell lung cancer patients: a pilot study with 3'-deoxy-3'-[(1)(8)F]fluorothymidine positron emission tomography. PLoS One. 2013; 8:e63705.

21. Hong YS, Kim HO, Kim KP, Lee JL, Kim HJ, Lee SJ, Lee SJ, Oh SJ, Kim JS, Ryu JS, Moon DH, Kim TW. 3'-Deoxy-3'-18F-fluorothymidine PET for the early prediction of response to leucovorin, 5-fluorouracil, and oxaliplatin therapy in patients with metastatic colorectal cancer. J Nucl Med. 2013; 54:1209-1216.

22. Takezawa K, Okamoto I, Okamoto W, Takeda M, Sakai K, Tsukioka S, Kuwata K, Yamaguchi H, Nishio K, Nakagawa K. Thymidylate synthase as a determinant of pemetrexed sensitivity in non-small cell lung cancer. Br J Cancer. 2011; 104:1594-1601.

23. Sigmond J, Backus HH, Wouters D, Temmink OH, Jansen G, Peters GJ. Induction of resistance to the multitargeted antifolate Pemetrexed (ALIMTA) in WiDr human colon cancer cells is associated with thymidylate synthase overexpression. Biochem Pharmacol. 2003; 66:431-438.

24. Saga Y, Suzuki M, Mizukami H, Kohno T, Takei Y, Fukushima M, Ozawa K. Overexpression of thymidylate synthase mediates desensitization for 5-fluorouracil of tumor cells. Int J Cancer. 2003; 106:324-326.

25. Copur S, Aiba K, Drake JC, Allegra CJ, Chu E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol. 1995; 49:1419-1426.

26. Patki M, Gadgeel S, Huang Y, McFall T, Shields AF, Matherly LH, Bepler G, Ratnam M. Glucocorticoid receptor status is a principal determinant of variability in the sensitivity of non-small-cell lung cancer cells to pemetrexed. J Thorac Oncol. 2014; 9:519-526.

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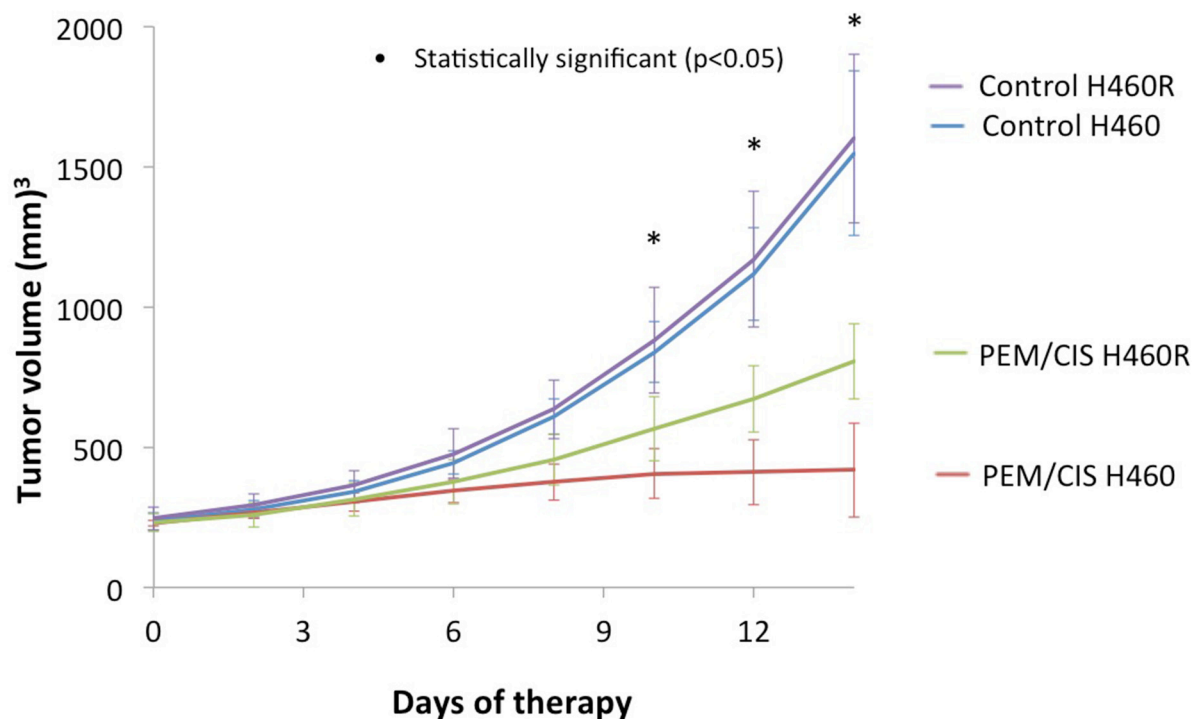
SUPPLEMENTARY FIGURES

Supplementary Figure 1: Thymidylate synthase expression in NSCLC cell lines. TS protein expression was lower in the pemetrexed sensitive lines, H460 and H1299, relative to the remainder of the tested NSCLC cell lines which were resistant to pemetrexed.

NSCLC cell line sensitivity to pemetrexed

Cell lines	IC50 (µM)	Cell lines	IC50 (µM)
H460	0.141	H460R	22.8
H1299	0.656	H1299R	213
H23	0.221	CALU6	14.2
H1975	0.731	H522	59.6
H1703	0.618	H1838	121
HCC827	0.175	H661	85.0
H1650	0.182	H650	238
H1155	0.402	H820	48.0

Supplementary Figure 2: Pemetrexed sensitivity of NSCLC cell lines. The IC50 was calculated for each NSCLC cell line.

Supplementary Figure 3: Overexpression of TS results in decreased tumor growth inhibition relative to wild-type pemetrexed H460 *in vivo*. H460 and H406R bearing xenografted mice were either treated as controls or given combination therapy with cisplatin and pemetrexed (PEM/CIS). By day 14 of therapy, H460R xenografts exhibited significantly less tumor growth inhibition compared to the wild-type H460 xenografts, although both groups had significant tumor growth inhibition compared to the untreated controls.

Cancer Research

Tumor Biology

Abstract 4233: Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small lung cancer with FLT-PET imaging

Xiao Chen, Ian Berger, Urooj Khalid, Jenny Cai, Akash Patel, and Sharyn I. Katz

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Proceedings: AACR 107th Annual Meeting 2016; April 16-20, 2016; New Orleans, LA

Abstract

Introduction: Successful inhibition of thymidylate synthase (TS) can be imaged with FLT ([18F]thymidine)-positron emission tomography (PET) thereby providing a measure of therapy response on day 1 of therapy. Here we characterize this imaging strategy for implementation into 1st line therapy with pemetrexed and cisplatin for NSCLC.

Materials and Methods: The kinetics of pemetrexed-mediated TS inhibition mediated DNA salvage pathway "flare" in DNA was first characterized in vitro using 3H-thymidine DNA incorporation assays, analysis of TK1 protein expression and mobilization of the equilibrative nucleoside transporter 1 to the cell surface membrane. Kinetics of the pemetrexed-mediated "flare" in the salvage pathway was then confirmed in an in vivo mouse model of NSCLC. Finally, a proof-of-principle clinical trial was performed to demonstrate feasibility of imaging of pemetrexed-mediated TS inhibition in human subjects with NSLC.

Results: In vitro examination of pemetrexed-induced changes in the salvage pathway revealed a burst in DNA activity that peaked at 2 hrs following the administration of pemetrexed. The addition of cisplatin did not impact the amplitude or timing of the pemetrexed-induced "flare" in the salvage pathway. In vivo imaging of TS inhibition

with FLT-PET confirmed a peak in salvage pathway activity at 2 hrs. Imaging of pemetrexed-induced TS inhibition in a human clinical trial demonstrated feasibility with imaging at the 2 hr time point.

Conclusion: FLT-PET measured efficacy of pemetrexed-induced TS inhibition is optimal at 2 hrs from the start of therapy. This timing of FLT-PET imaging is feasible in human clinical trials.

Citation Format: Xiao Chen, Ian Berger, Urooj Khalid, Jenny Cai, Akash Patel, Sharyn I. Katz. Early detection of pemetrexed-induced inhibition of thymidylate synthase in non-small lung cancer with FLT-PET imaging. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 4233.

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Table of Contents
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Abstract 1783: Early detection of thymidylate synthase resistance in non-small cell lung cancer with FLT-PET imaging

Xiao Chen, Yizeng Yang, and Sharyn Katz

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Abstract

Background: Inhibition of thymidylate synthase (TS) results in a transient compensatory "flare" in thymidine salvage pathway activity which is measureable with ^{18}F-thymidine (FLT) positron emission tomography (PET) at 2 hrs of exposure to therapy. Here we examine the predictive value of the FLT-PET measured "flare" for NSCLC sensitivity to pemetrexed, a commonly used TS inhibitor, in a preclinical model.

Experimental Design: Resistance to pemetrexed therapy was induced in two sensitive human NSCLC cell lines, H460 and H1299, by overexpressing TS. TS overexpression was confirmed with RT-PCR and Western blotting and pemetrexed-resistance confirmed with IC_{50} assays. The presence of a pemetrexed-induced DNA salvage pathway "flare" was then measured using ^{3}H-thymidine in both pemetrexed-sensitive (H460 and H1299) and resistant (H460R and H1299R) lines in vitro, as well as inherently resistant NSCLC cell lines CALU6, H522, H650, H661, H820, H1838, and validated with FLT-PET in vivo using H460 and H460R xenografts.

Results: Overexpression of TS induced resistance to pemetrexed in the H460 and H1299 with IC50 for H460, H1299, H460R and H1299R measured as 0.141 μM, 0.656 μM, 22.842 μM, 213.120 μM, respectively. Significant increases in DNA salvage pathway activity ("flare") was observed at 2hrs of therapy in the pemetrexed-sensitive

H460 and H1299 lines but not the resistant H460R and H1299R, CALU6, H522, H650, H661, H820, H1838 cell lines in vitro using ^{3}H-thymidine assays. Similarly, FLT "flare" was observed in the pemetrexed-sensitive H460 xenograft tumors but not the pemetrexed-resistant H460R xenograft tumors with post-therapy increases in measured FLTmax of 58% (STD 12.1) and 10.8% (STD 7.3) respectively.

Conclusions: FLT-PET imaging of TS inhibition may provide an early indicator of NSCLC sensitivity to pemetrexed.

Citation Format: Xiao Chen, Yizeng Yang, Sharyn Katz. Early detection of thymidylate synthase resistance in non-small cell lung cancer with FLT-PET imaging [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2017;77(13 Suppl):Abstract nr 1783. doi:10.1158/1538-7445.AM2017-1783

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